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Zinc in Human Health



Edited by
Lothar Rink

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ZINC IN HUMAN HEALTH

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This book is dedicated to my mentor Prof. Dr. Holger Kirchner, who encouraged me to start zinc research 20 years ago.

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Foreword

A little more than ten years ago, I travelled from Pittsburgh to the Cayman Islands to attend a “Zinc Signals” conference organized by Chris Frederickson. Chris had invited me, then a relative newcomer to the field, to present the work Ian Reynolds and I had recently published on the role of intracellular zinc release in neuronal cell death, to a gathering of approximately thirty or forty scientists that worked primarily on the biology of zinc. That meeting was a true eye-opener for me. For nearly a week, I was surrounded by chemists, immunologists, yeast biologists, and countless other specialists, all brought together by their devotion to the biology of zinc – all wonderfully willing to share their insights, reagents, and a good time snorkeling in the reef known as Stingray City. I made many new friends there, and, importantly, established several new collaborations that, to this day, continue to evolve as well as enrich my scientific life in countless ways. It was in Cayman I became an official zinc scientist.

In 2008, with Glen Andrews leading the way, we established the International Society for Zinc Biology, over which I have the honor of currently presiding. After a highly successful gathering that year of approximately 140 zinc scientists in Banff, Canada, and again in Jerusalem, Israel nearly two years later, we are now in the midst of preparing for our third official conference as a society, to be held in Melbourne, Australia in January 2012.

And now this very timely book! My colleague Lothar Rink has assembled a rich collection of chapters that represent the very essence of the field, a snapshot of the brilliant convergence of medicine and basic science in the study of zinc in human health and, importantly, disease. Leaders of their respective fields have made insightful and thorough contributions to this book, covering topics that range from human nutrition to Alzheimer’s disease, from pregnancy to aging, from the digestive system to the brain. To the “zincophile”, the book will offer an intellectually rewarding, scholarly collection of the most important components that constitute the field of zinc biology as it currently stands. To the newcomer, this book represents an opportunity to learn what the field has been up to since Ananda Prasad firmly established zinc as an essential nutrient in humans more than fifty years ago. However, be forewarned, this is a fast-moving field that has finally reached a critical mass of able and productive scientists. The future of zinc research is now here. Enjoy the ride, and as always, think zinc!

Elias Aizenman, Ph.D.
President, International Society for Zinc Biology
Pittsburgh, PA, U.S.A. March 2011

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I. INTRODUCTION AND PHYSIOLOGICAL BACKGROUND

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1. Introduction – Why Investigate Zinc?

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Abstract. Zinc is an essential trace element. The human body contains 2-3 g of zinc. Since there are no storing systems for zinc in the body, zinc has to be ingested daily and the homeostasis has to be regulated accurately. The recommended daily intake varies between 7-12mg of zinc. The chemical properties of zinc made it to one of the favorite metal ions in biological processes. This results in hundreds of zinc-dependent enzymes and signaling events dependent on zinc. Due to this importance a deficiency in zinc is related to multiple organ dysfunctions. Since zinc deficiency has been described in humans it became more and more obvious that severe zinc deficiency is one of the leading health problems in developing countries and marginal zinc deficiency a common problem in the industrial world. Therefore a detailed knowledge about zinc is necessary to understand dysfunctions in zinc homeostasis and to develop therapies using the biological capacity of this trace element.

Keywords. RDA; Zinc homeostasis; Zinc deficiency; Zinc intoxication; DALY

Introduction

Zinc was first described as an essential trace element by Raulin in 1869 [reviewed in 1]. However, it took nearly one century until Dr. Prasad could prove the existence of zinc deficiency in man in 1963 (see chapter 2 for details) [2]. After this breakthrough the knowledge about zinc evolved rapidly and especially the last two decades introduced the molecular background of the essentiality of this ion. When zinc deficiency was discovered it was thought to be a rare disease, but now we know, that zinc deficiency is one of the leading life-threatening factors, especially in developing countries (table 1) [3].

Table 1. Selected factors of the 20 leading risk factors for the loss of healthy life years.

Given are the percent of DALY (disability-adjusted life years) of the global DALY. DALY are the sum of life losses and years lost due to disability, giving a value of the loss of healthy life years of a population [3].

Rank number/Risk factor	% of global DALY	% of DALY in developing countries with high mortality (adapted position of risk factor)
1. Underweight	9.5	15.0 (1)
4. Tobacco	4.0	2.0 (9)
5. Alcohol	4.0	
7. Cholesterol	2.8	1.8 (10)
9. Iron deficiency	2.5	3.3 (6)
10. Overweight	2.4	
11. Zinc deficiency	2.0	3.5 (5)
12. Low fruit and vegetable intake	1.9	
13. Vitamin A deficiency	1.8	3.0 (7)

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However, also in developed countries zinc deficiency becomes more and more prevalent in the ageing community, where up to 42.5% show a marginal zinc deficiency [2]. The Food and Agriculture Organization (FAO) calculated on the bases of the worldwide food supply that half of the world population is at risk for zinc deficiency [4].

The total amount of zinc in the human body is about 2-3g [2], but the zinc content varies considerably between tissues, (table 2) [5-7]. Plasma zinc concentrations are only 10-18 μ M (around 1 μ g/g) [1]. Cellular zinc is mostly bound to proteins. The fact that more than 300 enzymes are dependent on zinc makes this ion indispensable for a multitude of cellular functions and organ systems [8]. and an even higher number of other proteins were found to contain zinc. Genome wide searches estimate that up to 10% of human proteins may contain zinc [2].

Table 2. Distribution of zinc in the human body [5-7].

Tissue	Zinc concentration per tissue wet weight [μ g/g]
Prostate	84-211
(Hyperplasia)	(760)
(Carcinoma)	(46)
Hair	150
Pancreas	140
(Diabetic)	(70)
Bone	100
Liver	58
Kidney	55
Muscle	51
Skin	32
Heart	23
Brain	11
Plasma	1

Although this clearly indicates that zinc is one of the most important trace elements for human well being, our knowledge about the requirements are still unsatisfactory. The recommended daily intake of zinc varies in different countries (table 3), which is one indicator of a lack of basic research. However, this may also be related to the multitude of factors influencing zinc uptake, which will be highlighted in chapter 3. Furthermore, zinc supplements have never been compared in a comprehensive study and the discussion about the best formula is still in progress, but organic compounds seem to be the best [4].

Table 3. Recommended daily intake of zinc in mg/day.

The recommended daily allowance (RDA) for zinc varies worldwide. Given are the detailed values for USA and (Germany) as well as the values for adults recommended in the European Union (EU), United Kingdom (UK), the World Health Organization (WHO) and indicated countries.

Age	Male	Female	Pregnant	Lactating
0-6 Months	2 (1)	2 (1)		
7-12 Months	3 (2)	3 (2)		
1-3 Years	3 (3)	3 (3)		
4-8 Years	5 (5)	5 (5)		
9-13 Years	8 (9)	8 (7)		
14-18 Years	11 (10)	9 (7)	12	13
19+ Years	11 (10)	8 (7)	11 (10)	12 (11)
EU	10	10		
Ireland	10	10		
Norway	9	7		
UK	9.5	7		
WHO	11	8		

Irrespective of these basic problems our knowledge about the role of zinc increased dramatically during the last decades. Therefore this book is intending to give a state-of-the-art of our knowledge of zinc in human health, since the last comprehensive textbook about zinc is more than 20 years old [5]. After introducing zinc research in the first 3 chapters, the following chapters will describe the function of zinc on the cellular and molecular level (figure 1). This will introduce the general knowledge about zinc in molecular and cellular processes which are important in all organ systems.

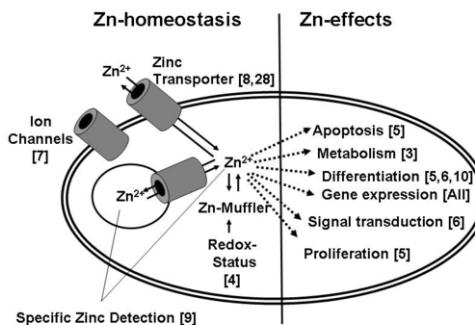


Figure 1: Molecular and cellular effects of zinc. Shown are the main functions of zinc on the cellular level. The numbers indicate the appropriate chapter where the particular effect will be described in detail.

After this general background, the specific role of zinc in different organ systems will be described (figure 2). All organ systems are included where a number of publications have shown a detailed view on zinc in health and disease of this organ system. However, zinc also influences other organ systems not covered in this text book, but the current knowledge is too small to give information above the general cellular effects described before.

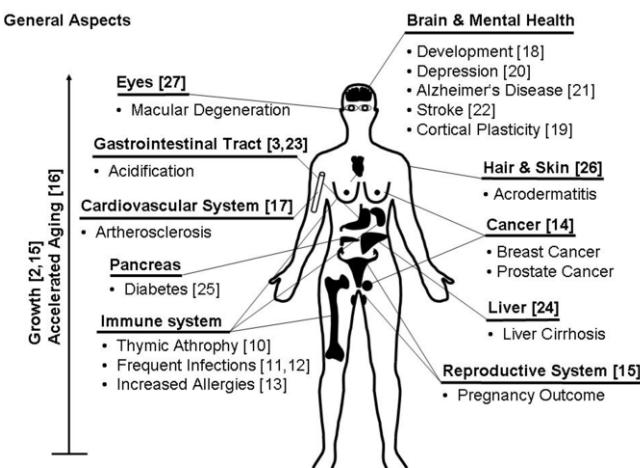


Figure 2: General aspects of zinc on the function of different organ systems. Shown are the main functions of zinc on the organ systems as well as general factors as growth and ageing. The numbers indicate the appropriate chapter where the particular organ system will be described in detail.

However, beside the specific organ systems zinc is very important during development and ageing. Both will be discussed in separate chapters to show the importance especially during childhood, which is the main problem in developing countries, and during ageing, which is the main problem in the industrial world.

Before the reader starts with this textbook some general remarks have to be made. Whenever zinc is described the Zn²⁺ ion is meant, since zinc as a transition element exists only in this oxidation state. Elementary zinc does not have any biological function, it is only the Zn²⁺ ion. Furthermore, different terms are used as “available”, “loosely bound”, “exchangeable”, “chelateable”, “flexible” or “free” zinc. All terms describe the free cytoplasmic or extracellular zinc which can mediate the effects due to a fast transfer to active sites of enzymes and other proteins. Therefore free zinc is the best term to use, but all terms are used in this book.

The reader may also wonder, why no chapter about zinc toxicology is given, since zinc is a heavy metal. The number of well described zinc intoxications are rare. There are some cases where schizophrenic patients ingested high amounts of zinc by swallowing a high number of one cent coins [2] and one case of a patient eating 60 oysters [1], which may contain more than only zinc. The first cases lead to copper deficiency, whereas the second showed an acute syndrome. Acute syndromes are also known from metal-working causing the metal (zinc) fume fever [1]. However, recently a common series of zinc induced copper deficiency was described in the United States due to excessive use of zinc-based denture adhesive [9]. This problem was solved by the Food and Drug Administration (FDA) by limiting the amount of zinc in the denture adhesive.

Therefore zinc intoxication plays nearly no role in human health, whereas even marginal zinc deficiency plays an important role in human health, which will be described in detail in this textbook.

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2. Discovery of Zinc Deficiency in Humans and its Impact Fifty Years Later

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Abstract. Essentiality of zinc for humans and its deficiency was recognized in 1963. During the past 50 years, it has become apparent that deficiency of zinc in humans is widely prevalent. Nutritional deficiency of zinc may affect nearly 2 billion subjects in the developing world. Consumption of cereal proteins high in phytate decreases the bio-availability of zinc. Conditioned deficiency of zinc is also very common. Growth retardation, hypogonadism in males, rough skin, impaired immunity, neuro-sensory disorder and cognitive impairment are some of the major clinical manifestations of zinc deficiency. Zinc is involved in many biochemical functions and nearly 2000 transcription factors require zinc for gene expression. Zinc is also an effective antioxidant and anti-inflammatory agent. In therapeutic dosages, zinc has been used for the treatment of acute diarrhea decreasing mortality in millions of affected infants and children, common cold, Wilson's disease, sickle cell disease and for the prevention of blindness in patients with age related macular degeneration.

Keywords. Zinc, growth-retardation, immunity, IL-2, cognitive impairment, antioxidant, anti-inflammatory agent

Introduction

Raulin [1] in 1869 showed for the first time that zinc was essential for the growth of *Aspergillus niger*. In 1934 Todd et al. [2] reported that zinc was essential for the growth of the rats. In experimental zinc deficient animals the manifestations of zinc deficiency included growth failure, loss of hair, thickening and hyperkeratinization of the epidermis, and testicular atrophy. Although the essentiality of zinc for animals was established, its ubiquity made it seem improbable that zinc deficiency in humans could lead to significant problems in clinical medicine.

1. Discovery of Human Zinc Deficiency

I arrived in Shiraz, Iran, in June 1958 after finishing my formal training in medicine under Professor CJ Watson at the University of Minnesota, Medical School, Minneapolis, Minnesota. Dr Hobart A. Reimann, Chief of Medicine at the Nemazee Hospital of Pahlevi University in Shiraz, Iran, invited me to join him to set up a curriculum for teaching medicine to students and house staff. Professor Reimann was

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formally Chief of Medicine at Minnesota preceding Dr. C.J. Watson. In Shiraz, I met Dr James, A Halsted, who was a Fulbright Professor at Pahlevi University and was primarily involved with Saadi hospital, a hospital equivalent to city hospitals in USA. In the fall of 1958, I was invited by Dr Halsted to discuss a patient with anemia at the medical center grand rounds at the Saadi Hospital. The case was presented to me by the chief resident, Dr M Nadimi, a graduate of the Shiraz Medical School.

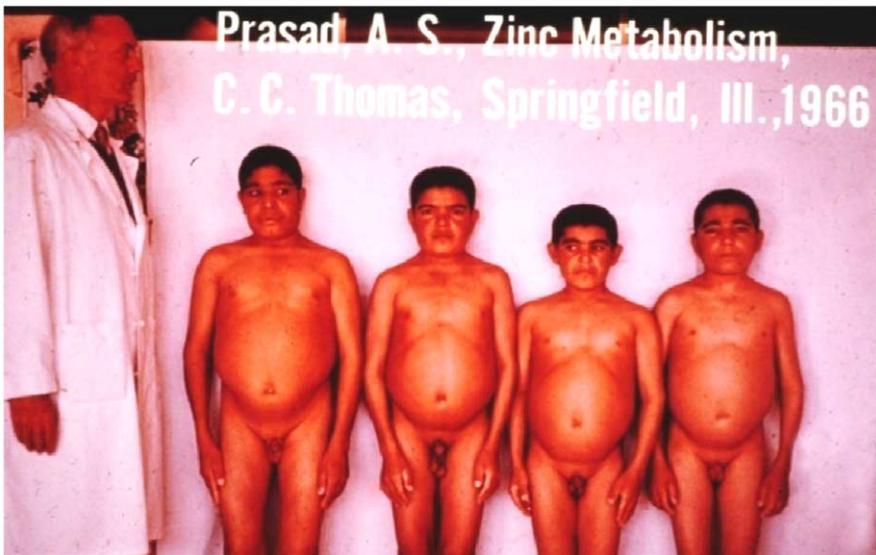


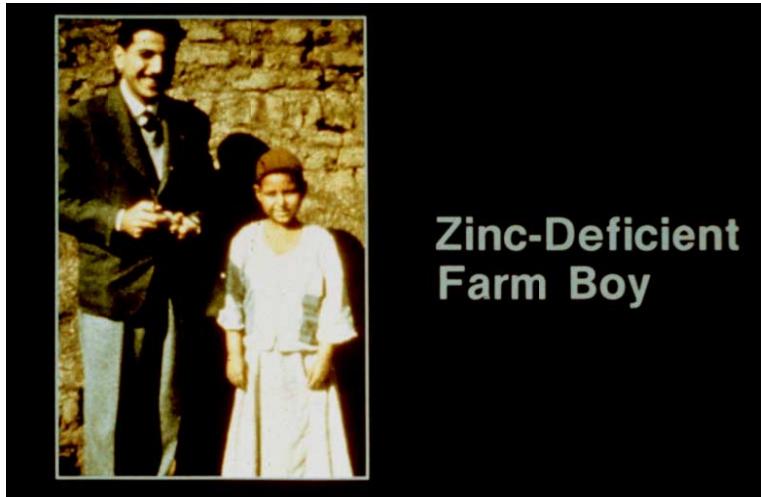
Figure 1. Zinc deficient Iranian dwarfs ages 16 to 21 years. (Ref.4)

The patient was a 21 -y-old male, who looked like a 10-y-old boy (fig. 1). In addition to severe growth retardation and anemia he had hypogonadism, hepatosplenomegaly, rough and dry skin, mental lethargy, and geophagia. The patient ate only bread made from whole wheat flour and his intake of animal protein was negligible. He consumed nearly 0.5 kg of clay daily. Later we discovered that the habit of geophagia (clay eating) was fairly common in the villages around Shiraz. Further studies documented the existence of iron- deficiency anemia in our patient but, there was no evidence of blood loss. Inasmuch as 10 additional similar cases were brought to the hospital for my care within a short period of time, hypopituitarism as an explanation for growth retardation and hypogonadism was ruled out.

The anemia of the subjects promptly responded to oral administration of iron. The probable factors responsible for anemia in these patients were poor availability of iron in the diet, excessive sweating probably causing greater iron loss from the skin than would occur in a temperate climate and geophagia further decreasing iron absorption.

It was difficult to explain all of the clinical features solely by iron deficiency inasmuch as growth retardation and testicular atrophy are not seen in iron-deficient experimental animals. We considered the possibility that zinc deficiency also may have been present. As noted earlier, zinc deficiency was known to produce retardation of growth and testicular atrophy in animals. Because heavy metals may form insoluble complexes with phosphate, we speculated that some factors responsible for decreased availability of iron in these patients with geophagia may also have decreased the availability of zinc. O'Dell and Savage [3] had observed that phytate (inositol

hexaphosphate), which is present in cereal grains, markedly impaired the absorption of zinc.



Zinc-Deficient
Farm Boy

Figure 2. Zinc-deficient Egyptian dwarf age 19 years (right). On the left is the house physician. (Ref. 5)

We published a clinical description of the Iranian cases as a syndrome and speculated that zinc deficiency may account for growth retardation and male hypogonadism in these subjects [4]. I left Iran in January 1961 and joined the department of Biochemistry and Medicine at Vanderbilt University under Dr. William J Darby. Although Dr. Darby wanted me to study porphyrin metabolism in Pellagra in Egypt, I shared with him my speculation that zinc deficiency in the Middle East was prevalent and was responsible for widespread growth retardation. He approved my plans to investigate zinc metabolism in growth-retarded subjects. I then moved to US Naval medical research unit No.3 (NAMRU-3) at Cairo, Egypt. My team consisted of Harold Sandstead, MD, and A. Schulert, PhD, both from Vanderbilt University, A. Miale Jr. MD from NAMRU-3 and Z. Farid, MD, a local physician, also from NAMRU-3.

In Egypt subjects similar to the growth-retarded Iranian subjects were encountered (fig. 2). The clinical features were remarkably similar except that the Iranian subjects had more pronounced hepatosplenomegaly, a history of geophagia, and no hookworm infection and the Egyptian subjects had both schistosomiasis and hookworm infestations but no history of geophagia.

We carried out a detailed investigation of the Egyptian cases at NAMRU-3 in Cairo. The dietary history of the Egyptian subjects was similar to that of the Iranians. Their diet consisted mainly of bread and beans (*Vicia fava*) and intake of animal protein was negligible. These subjects were shown to have zinc deficiency. The evidences were decreased zinc concentrations in plasma, red cells, and hair and zinc-65 studies revealed, that the plasma zinc turnover was greater, the 24-h exchangeable pool was smaller, and the excretion of zinc-65 in stool and urine was less in the zinc deficient subjects than in the control Subjects [5].

Hypozincemia in humans in the absence of advanced cirrhosis of the liver had not been described before. Liver-function tests and biopsy in these subjects revealed no

evidence of cirrhosis. Furthermore, in contrast to cirrhosis patients who excrete abnormally high quantities of zinc in urine, our patients excreted less zinc in urine than the control subjects. Other chronic debilitating diseases that might affect the serum zinc concentrations were ruled out.

It was a common belief among clinicians in Iran that severe growth retardation and sexual hypofunction, as noted in these subjects, were the results of visceral leishmaniasis and geophagia. In our studies no evidence of visceral leishmaniasis was found. The role of geophagia was not entirely clear; however, it was suspected that the excess amount of phosphate in the clay may have prevented absorption of both dietary iron and zinc. The predominantly wheat diet in the Middle East, now known to contain high quantities of phytate and fiber, most probably reduced the availability of zinc. In Egypt, the cause of dwarfism was commonly considered to be schistosomiasis. Chinese investigators had also implicated schistosomiasis as a causative factor for growth retardation.

Our studies in the Middle East only included males. Female subjects refused to participate in our studies. Later studies from Iran by Halsted et al. [6] demonstrated that zinc deficiency in females affected growth and ovarian functions adversely.

Supplementation studies in Egypt showed that the rate of growth was greater in patients who received zinc as compared with those receiving iron instead or those receiving only an adequate animal-protein diet [7]. Pubic hair appeared in all subjects within 7-12 wk after zinc supplementation. Genitalia increased to normal size and secondary sexual characteristics developed within 12-24 weeks in patients who received zinc [7]. In contrast, no such changes were observed in a comparable length of time in the iron-supplemented group or in the group on an animal-protein diet. Thus, the growth retardation and gonadal hypofunction in these subjects were related to zinc deficiency. The anemia was due to iron deficiency and responded to oral iron treatment.

Zinc deficiency in human populations throughout the world is prevalent. Clinical pictures similar to those reported in zinc-deficient dwarfs have been observed in many countries. It is believed that zinc deficiency should be present in countries where primarily cereal proteins are consumed by the population. One would also expect to see a spectrum of zinc deficiency, ranging from severe cases to marginally deficient examples, in any given population.

In 1968, MacMahon et al. [8] observed for the first time, zinc deficiency in a patient who had steatorrhea and in 1972 Hambidge et al. [9] reported occurrence of zinc deficiency in Denver children.

In 1973, Barnes and Moynahan [10] studied a 2-y-old girl with severe acrodermatitis enteropathica who was being treated with diiodohydroxyquinoline and a lactose-deficient synthetic diet. The clinical response to this therapy was not satisfactory and the physicians sought to identify contributing factors. The concentration of zinc in the patient's serum was profoundly decreased; therefore, they administered oral zinc sulfate. The skin lesions and gastrointestinal symptoms cleared completely. When zinc was inadvertently omitted from the child's regimen, she suffered a relapse; however, she again promptly responded to oral zinc. In their initial reports the authors attributed zinc deficiency in this patient to the synthetic diet. It soon became clear that zinc might be fundamental to the pathogenesis of this rare inherited disorder and that the clinical improvement reflected improvement in zinc status. This original observation was quickly confirmed in other patients throughout the world. The underlying pathogenesis of the zinc deficiency in these patients is due to malabsorption of zinc due to a mutation in ZIP4, a zinc transporter [11].

In 1974 a landmark decision to establish recommended dietary allowances (RDAs) for humans for zinc was made by the Food and Nutrition Board of the National Research Council of the USA National Academy of Sciences.

2. Chronology of Other Observations

Lutz (1926), by using dithizone technique, assayed zinc in various tissues and calculated the total zinc content of a 70 Kg man as being 2.2 g, a figure remarkably close to what is accepted today [12]. McCane and Widdowson (1942) were the first to report on the absorption and excretion of zinc in humans and showed that the principal route of zinc excretion was in the feces and only a small amount was excreted in the urine [13].

Vikbladh (1950) measured serum zinc concentration by dithizone technique and reported that the level was $19.7 \pm 0.24 \mu\text{mol/L}$, a value in general agreement with those reported by using modern methods [14]. Vikbladh (1951) also observed that serum zinc concentration was decreased in many chronic diseases, including liver disease [15]. Vallee et al. (1956) reported that the serum zinc concentration was decreased in patients with cirrhosis of the liver and suggested that these subjects had conditioned deficiency of zinc due to hyperzincuria [16].

In the United States, Caggiano et al. (1969) were the first to report a case of zinc deficiency in a Puerto Rican subject with dwarfism, hypogonadism, hypogammaglobulinemia, giardiasis, strongyloidosis, and schistosomiasis [17]. Zinc supplementation resulted in improved growth and development. Our recent studies have shown that in the U.S.A., zinc deficiency in the well-to-do elderly may be fairly prevalent [18]. Thus, it is obvious that the risk of suboptimal zinc nutrition may pose a problem for a substantial section of the U.S. population.

Halsted et al. [6] published the results of their study involving a group of 15 men who were rejected at the Iranian Army Induction Center because of "malnutrition." Two women, 19 and 20 years old, were also included in their study. A unique feature was that all were 19 or 20 years old. Their clinical features were similar to those reported earlier by Prasad et al. [4, 5]. They were studied for 6-12 months. One group was given a well-balanced diet containing ample animal protein plus a placebo capsule. A second group was given the same diet plus a capsule of zinc sulfate containing 27 mg zinc. A third group received the diet without additional supplement for 6 months, followed by the diet plus zinc for another 6 months. The two women lived in the house of Dr. Ronaghy and received the same treatment and observation program.

The zinc supplemented subjects grew considerably faster and showed evidence of early onset of sexual function, as defined by nocturnal emission in males and menarche in females, than those receiving the well-balanced diet alone [6].

Kay and Tasman-Jones in 1975 [19] reported the occurrence of severe zinc deficiency in subjects receiving total parenteral nutrition for prolonged periods without zinc. Okada et al. [20] and Arakawa et al. [21] reported similar findings in subjects receiving total parenteral nutrition without zinc. These observations have been documented by several investigators and, indeed, in the United States, zinc is being routinely included in total parenteral fluids for subjects who are likely to receive such therapy for extended periods.

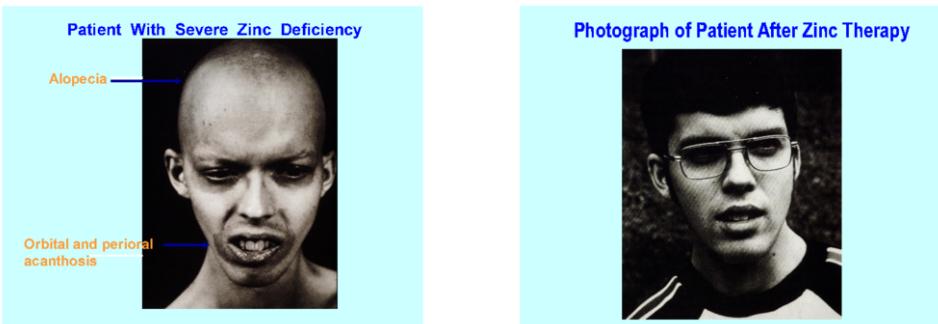


Figure 3. An example of severe zinc deficiency induced by penicillamine therapy in a patient with Wilson's disease. (Ref. 30)

Figure 4. Improvement following zinc supplementation. Same patient as Figure 3. (Ref.30)

Klingberg et al. in 1976 [22] were the first to report severe parakeratosis, alopecia, retardation of growth and gonadal development in an adolescent with Wilson's disease who received penicillamine therapy. Zinc supplementation completely reversed the clinical manifestations.

3. Clinical Effects of Zinc Deficiency

3.1. Clinical Spectrum of Human Zinc Deficiency

During the past five decades, a spectrum of clinical deficiency of zinc in human subjects has been recognized. On the one hand, the manifestations of zinc deficiency may be severe; and, on the other end of the spectrum, zinc deficiency may be mild or marginal. A severe deficiency of zinc has been reported to occur in patients with acrodermatitis enteropathica, following total parenteral nutrition (TPN) without zinc, following excessive use of alcohol, and following penicillamine therapy.

3.2. Acrodermatitis Enteropathica

Acrodermatitis enteropathica (AE) is a lethal, autosomal, recessive trait that usually occurs in infants of Italian, Armenian, or Iranian lineage [10]. This disease is not present at birth but usually develops in the early months of life soon after weaning from breast feeding. The dermatologic manifestations of severe zinc deficiency in patients with AE include bullous pustular dermatitis of the extremities and the oral, anal, and genital areas (around the orifices) combined with paronychia and generalized alopecia. Ophthalmic signs may include blepharitis, conjunctivitis, photophobia, and corneal opacities. Neuropsychiatric signs include irritability, emotional disorders, tremors, and occasional cerebellar ataxia. The patients with AE generally have weight loss, growth-retardation; and males exhibit hypogonadism. A high incidence of congenital malformation of fetuses and infants born of pregnant women with AE has been reported [23].

Patients with AE have an increased susceptibility to infections. In AE, thymic hypoplasia, absence of germinal centers in lymph nodes and plasmacytosis in the

spleen are found consistently. All T cell mediated functional abnormalities are completely corrected with zinc supplementation. Abnormal chemotaxis correctable with zinc therapy has also been reported in AE patients. In general, the clinical course is downhill with failure to thrive and complicated by intercurrent bacterial, fungal, and other opportunistic infections. Gastrointestinal disturbances are usually severe, including chronic diarrhea, malabsorption, steatorrhea, and lactose intolerance. The disease, if unrecognized and untreated, is fatal. Zinc supplementation results in complete recovery.

AE gene has been localized to a ~3.5-cM region on 8 q 24. The gene encodes a histidine-rich protein, which is now referred to as hZIP-4, which is a member of a large family of transmembrane proteins, some of which are known to serve as zinc-uptake proteins (see chapter 8). In patients with AE, mutations in this gene have been documented [11].

3.3. Total Parenteral Nutrition (TPN)

Patients on TPN with diarrhea may lose 6 to 12 mg of zinc/d. This excessive loss of zinc may result in a severe deficiency of zinc. In such cases not only dermatologic manifestations are seen but also alopecia, neuro psychiatric manifestations, weight loss, and intercurrent infections, particularly involving opportunistic infections are also observed. Carbohydrate utilization is impaired, and there is a negative nitrogen balance. If zinc deficiency in such cases is not recognized and treated, the condition may become fatal.

In summary, the manifestations of severe zinc deficiency in humans include bullous pustular dermatitis, alopecia, diarrhea, emotional disorder, weight loss, intercurrent infections due to cell mediated immune dysfunctions, hypogonadism in males, neuro-sensory disorders, and problems with healing of ulcers. If this condition is unrecognized and untreated, it becomes fatal.

3.4. Moderate Deficiency of Zinc

A moderate level of zinc deficiency has been reported in a variety of conditions. These include nutritional due to dietary factors, malabsorption syndrome, alcoholic liver disease, chronic renal disease, sickle cell disease, and chronically debilitated conditions.

3.4.1. Nutritional

Growth retardation, hypogonadism in the males, poor appetite, mental lethargy, rough skin, and intercurrent infections were the classical clinical features of chronically zinc deficient subjects from the Middle East as reported by Prasad et al. in the early 1960s [5,6]. The basis for zinc deficiency was nutritional inasmuch as zinc was poorly available from their diet due to high content of phytate and phosphate. All the above mentioned features were corrected by zinc supplementation.

As mentioned before, it is now apparent that a nutritional deficiency of zinc in humans is fairly prevalent throughout the world, particularly in areas where cereal proteins are primary in local diet. Just as in Iran, in Turkey also geophagia is a common problem and the majority of the adolescents with geophagia exhibit both iron and zinc deficiencies.

Cavdar et al. [24] observed a decreased zinc level in almost 30 percent of pregnant women in Turkey, all of whom were of low socioeconomic status. Their diet consisted mainly of cereals. In view of the serious teratogenic effects of maternal zinc deficiency in experimental animals as well as epidemiological evidence that maternal zinc deficiency could be a factor responsible for severe congenital malformation of the central nervous system in humans, correction of this nutritional problem in pregnant women are urgently needed (see chapter 15).

3.4.2. Gastrointestinal Disorders and Liver Disease

A moderate level of zinc deficiency has been observed in many gastrointestinal disorders. These include malabsorption syndrome, Crohn's disease, regional ileitis, and steatorrhea. A low serum and hepatic zinc and, paradoxically, hyperzincuria was demonstrated in patients with cirrhosis of the liver many years ago.

Some patients with cirrhosis of the liver who had night blindness, did not respond to Vitamin A therapy, however, an improvement following zinc supplementation was reported. Hepatic coma may be precipitated by administration of methionine to cirrhosis patients with an Eck fistula. Similarly, elevated blood ammonia seems to be intimately related to the development of hepatic coma. It is known that zinc-deficient rats have a defect in the metabolism of sulphur-containing amino acids. Zinc deficiency also affects urea synthesis and, thus abnormalities related to metabolism of amino acids and ammonia may act in concert to produce hepatic coma. We have reported an elevated level of plasma ammonia in human subjects as a result of dietary zinc restriction [25]. Rabbani and Prasad [26] observed a decrease in hepatic ornithine transcarbamoylase (OCT) activity and an increase in plasma ammonia levels in zinc-deficient rats. An increased activity of the purine nucleotide enzyme adenosine monophosphate deaminase (AMP-deaminase) as a result of zinc deficiency has also been observed; and it is possible that several factors may account for increased plasma ammonia levels in zinc deficiency associated with cirrhosis of the liver. Zinc therapy has been reported to be beneficial in subjects with hepatic encephalopathy. More studies are needed in this important area.

It is likely that some of the clinical features of cirrhosis of the liver, such as loss of body hair, testicular hypofunction, poor appetite, mental lethargy, difficulty in healing, abnormal cell mediated immune functions, and night blindness, may indeed be related to the secondary zinc-deficient state in this disease (see chapters 23 and 24).

3.4.3. Renal Disease

Mahajan et al. [27] first documented that patients with chronic renal failure had low concentrations of zinc in plasma, leukocytes, and hair as well as increased plasma ammonia levels and increased activity of plasma ribonuclease. Uremic hypoguesia improved following zinc supplementation. Impotence is common in uremic males and is not improved by hemodialysis (HD). A double-blind clinical trial of zinc supplementation was carried out using zinc acetate to determine the effect of zinc on uremic gonadal dysfunction [28]. The results of this study suggested that zinc deficiency was a reversible cause of sexual dysfunction in uremia.

3.4.4. Zinc Deficiency in Sickle Cell Disease

Our studies have documented the occurrence of zinc deficiency in adult sickle cell anemia (SCA) patients [29, 30]. Growth retardation, hypogonadism in males, hyperammonemia, abnormal dark adaptation, and cell mediated immune disorder in SCA has been related to a deficiency of zinc. The biochemical evidences of zinc deficiency in SCA included a decreased level of zinc in the plasma, erythrocytes, and hair, hyperzincuria and decreased activities of certain zinc dependent enzymes such as carbonic anhydrase in the erythrocytes, alkaline phosphatase in the neutrophils, deoxythymidine kinase activity in newly synthesizing skin connective tissue and collagen, and hyperammonemia. Inasmuch as zinc is known to be an inhibitor of ribonuclease (RNase), an increased activity of this enzyme in the plasma of SCA subjects was regarded as an evidence of zinc deficiency. Zinc supplementation to SCA subjects resulted in significant improvement in secondary sexual characteristics, normalization of plasma ammonia level, and reversal of dark adaptation abnormality. As a result of zinc supplementation, the zinc level in plasma, erythrocytes, and neutrophils increased, and an expected response to supplementation was observed in the activities of the zinc-dependent enzymes. We have also reported a beneficial effect of zinc on longitudinal growth and body weight in 14 to 18 year old patients with sickle cell anemia. Zinc deficiency in patients with sickle cell anemia was associated with impaired DTH (delayed type hypersensitivity reactions) and decreased NK (natural killer cells) cell lytic activity, which were corrected by zinc supplementation.

A three month placebo controlled zinc supplementation trial (25mg zinc as acetate three times a day) in 36 sickle cell disease patients showed that the zinc supplemented group had decreased incidence of infections, increased hemoglobin and hematocrit, plasma zinc and antioxidant power in comparison to the placebo group [31]. Plasma nitrite and nitrate (NO_x), lipid peroxidation products, DNA oxidation products, and soluble vacular cell adhesion molecule-1 decreased in the zinc supplemented group in comparison to the placebo group [31]. Zinc-supplemented subjects showed significant decreases in lipopolysaccharide-induced tumor necrosis factor-alpha (TNF- α) and interleukin (IL)-1 β mRNAs and TNF-induced nuclear factor of kB-DNA binding in mononuclear cells (MNCs) compared with the placebo group. Zinc supplementation also increased relative levels of IL-2 and IL-2R α mRNAs in phytohemagglutinine (PHA)-p stimulated MNCs.

In summary, the manifestations of a moderate deficiency of zinc include growth retardation and male hypogonadism in the adolescents, rough skin, poor appetite, mental lethargy, delayed wound healing, cell-mediated immune dysfunctions, and abnormal neurosensory changes (see chapter 10 for further details).

3.5. Mild Deficiency of Zinc

Although the clinical, biochemical, and diagnostic aspects of severe and moderate levels of zinc deficiency in humans are fairly well defined, the recognition of mild levels of zinc deficiency has been difficult. We therefore, developed an experimental model of zinc deficiency in order to define mild deficiency of zinc in humans. In a group of human volunteers we induced a mild state of zinc deficiency by dietary means. Adult male volunteers were hospitalized at the Clinical Research Center of the University of Michigan Medical School Hospital. A semi-purified diet, which supplied approximately 3.0 to 5.0 mg of zinc on a daily basis, was used to produce zinc deficiency [30].

The volunteers were ambulatory and were encouraged to do daily moderate exercise throughout the study period. Prior to the study, a thorough history, physical examination and routine laboratory tests such as CBC (peripheral blood total and differential cell count), liver function tests, SMA-12, and serum electrolytes were performed and found to be normal. Zinc in lymphocytes, granulocytes and platelets were determined and found to be in the normal range.

They were given a hospital diet containing animal protein daily for four weeks. This diet averaged 12 mg of zinc per day, consistent with the recommended dietary allowance of the National Research Council, National Academy of Sciences. Following this, they received 3.0 to 5.0 mg of zinc a day while consuming soy protein-based experimental diet. This regime was continued for 28 weeks, at the end of which two cookies containing 27 mg of zinc supplement was added to the experimental diet. The supplementation was continued for 12 weeks.

Throughout the study the level of all nutrients including protein, amino acids, vitamins, and minerals (both macro and micro elements) were kept constant meeting the standards set by RDA, except for zinc, which was varied as outlined above. By this technique we were able to induce a specific mild deficiency of zinc in human volunteers.

In our studies in the experimental human model in whom only a mild deficiency of zinc in males was induced by dietary means, decreased serum testosterone level, oligospermia, decreased NK cell activity, decreased IL-2 activity of T helper cells, decreased thymulin activity, hyperammonemia, hypogeausia, decreased dark adaptation, and decreased lean body mass were observed. It is, therefore, clear that even a mild deficiency of zinc in humans affects clinical, biochemical, and immunological functions adversely.

3.6. Major Clinical Effects of Zinc Deficiency in Humans

3.6.1. Growth Retardation

We documented zinc deficiency in growth retarded villagers in Egypt by the following criteria: the zinc concentrations in plasma, red cells and hair were decreased and the studies with ^{65}Zn in urine and stool was less in the growth retarded subjects than in the control subjects [6, 30]. Our studies also documented that the rate of growth was greater in patients who received zinc supplement as compared to those who received iron, or those who received only an adequate animal protein diet. Pubic hair appeared in all subjects within 7 to 12 weeks after zinc supplementation and genitalia increased to normal size and secondary sexual characteristics developed within 12 to 24 weeks in all subjects receiving zinc [7].

A meta-analysis of 33 studies of the effect of zinc supplementation on children's growth was reported by Brown et al. [32]. Prospective intervention trials were included if they enrolled a control group and provided suitable data on change in height or weight during the period of observation. The pooled study population included 1834 children less than 13 years of age, with representation from most regions of the world. The meta-analysis showed that in all children studied, zinc supplementation had a highly statistically significant positive response in height and weight increments respectively [32].

Nakamura et al. [33] screened 220 prepubertal subjects with short stature in a hospital clinic for zinc supplementation trial. In zinc supplemented children, caloric intake ($p < 0.01$), growth velocity ($p < 0.01$), serum zinc, calcium and phosphorus

concentrations, alkaline phosphatase activity ($p < 0.001$), percentage of tubular reabsorption of phosphorus ($p < 0.05$), ratio of maximal tubular reabsorption rate for phosphorus to the glomerular filtration rate ($p < 0.05$), serum osteocalcin level ($p < 0.01$), and plasma insulin like growth factor 1 (IGF-1) ($p < 0.05$) were significantly increased in comparison to control group but urinary excretion of growth hormone (GH) and other pituitary hormone levels in plasma were unchanged. Zinc supplementation resulted in a considerable increase in height. A significant elevation in serum IGF-1 level after zinc supplementation was observed. Increased plasma levels of IGF-1, despite unchanged GH production, may be explained if zinc has a role in binding GH to GH receptor in hepatic cells. Nakamura et al. [33] recommended that the children with non-endocrinologic short stature should be treated for at least six months with zinc, even if the serum zinc concentration is within normal range and zinc clearance test is not available. These children may have a marginal deficiency of zinc and they would respond to zinc supplementation with increase in height.

Approximately one-half of the children < 5 y of age in developing countries are retarded in statural growth [34]. Nutrient deficiencies underlie the stunting of growth in many of these children. Zinc deficiency has been associated with poor growth and zinc supplementation of growth-retarded children stimulated growth. Inasmuch as zinc deficiency is associated with cell-mediated immune dysfunctions, and since children with malnutrition show clinical evidences of immunologic deficit correctable by zinc [35], the high prevalence of infections in malnourished children might also be caused by zinc depletion [36].

Protein energy malnutrition, which affects 50% of Vietnamese children < 5 y of age, is apparent in children as young as 4-6 mo and reaches a peak frequency at the age of 2 y [37]. Zinc deficiency is associated with diets high in phytate and fiber and low in protein. Because $> 80\%$ of the dietary energy intake in Vietnam is derived from rice and intake of animal products is low, the Vietnamese diet places children at risk for zinc deficiency.

In order to determine whether zinc deficiency might be responsible for the failure to thrive observed in Vietnamese children, Ninh et al. [37] assessed growth, incidence of infections, and circulating IGF-1 concentrations in a double-blind study of zinc supplementation. Zinc supplementation increased weight ($+ 0.5 \pm 0.1$ kg; $p < 0.001$) and height ($+ 1.5 \pm 0.2$ cm; $p < 0.001$) after five months compared to placebo group. The relative risk of infectious episodes in the zinc supplemented group was reduced 3-fold for diarrhea ($p=0.012$) and 2.5-fold for respiratory tract infection ($p=0.057$). Plasma IGF-1 concentration increased in zinc treated subjects between one and five months ($p=0.018$), whereas no change in placebo group was observed.

This study showed that zinc deficiency was limiting growth in Vietnamese children and the growth stimulating effect of zinc might be mediated through changes in circulating IGF-1. Ninh et al. [38] investigated the mechanism responsible for the IGF-1 decline due to zinc deficiency in rats. Zinc depletion in comparison to pair-fed animals specifically reduced body weight gain (-22%, $p < 0.05$), serum IGF-1 concentration (-52%, $p < 0.001$), hepatic GH receptors (-28%, $p < 0.05$) and serum growth hormone binding protein (GHBp) levels (-51%, $p < 0.05$). Both zinc deficient and pair-fed groups of animals had reduced liver IGF-1 and GH receptor/GHBp mRNA levels in comparison with the adlibitum fed controls ($p < 0.01$). However, only liver IGF-1 mRNA levels were specifically reduced by zinc deficiency (zinc deficient vs pair-fed rats, $p < 0.05$). Zinc deficiency per se decreased serum IGF-1 independently of the reduction in food intake.

3.6.2. Zinc and Immunity

Zinc affects multiple aspects of the immune system [39-44] (see chapter 10). Zinc is crucial for normal development and function of cells mediating innate immunity, neutrophils and natural killer cells. Macrophages are also affected by zinc deficiency. Phagocytosis, intracellular killing, and cytokine production are all affected by zinc deficiency. Zinc deficiency also affects adversely the growth and function of T and B cells. This occurs through dysregulation of basic biological functions at the cellular level (see chapter 4). Zinc is needed for DNA synthesis, RNA transcription, cell division, and cell activation. Programmed cell death (apoptosis) is also potentiated in the absence of adequate levels of zinc (see chapter 5). Secretion and function of cytokines, the basic messengers of the immune system are adversely affected by zinc deficiency. The ability of zinc to function as an antioxidant and stabilize membranes suggests that it has a role in prevention of free radical induced injury during inflammatory processes.

It has been known for many years that zinc deficiency in experimental animals leads to atrophy of thymic and lymphoid tissue [39]. Later studies in young adult zinc deficient mice showed thymic atrophy, reductions in absolute number of splenocytes, and depressed responses to both T-cell-dependent (TD) and T cell-independent (TI) antigens [39].

A decrease in in vivo generated cytotoxic T killer activity to allogeneic tumor cells in zinc deficient mice and an impairment in cell mediated response to non-H₂ allogeneic tumor cells in zinc deficient mice have been reported [39]. Animals maintained on a zinc deficient diet for as little as 2 weeks developed a severe impairment in their ability to generate a cytotoxic response to the tumor challenge. This was totally reversible by zinc supplementation.

3.6.3 Studies of Immune Functions in Experimental Human Model

During our studies in the Middle East, we observed that most of the zinc deficient dwarfs did not live beyond the age of 25 years. The cause of death appeared to be infections. The possibility that zinc deficiency may have played a role in immune dysfunctions in the zinc deficient dwarfs was considered but lack of proper facilities prevented us from gathering meaningful data on immune functions in those patients.

We developed an experimental model, which allowed us to study specific effects of mild zinc deficiency in humans on immune functions [30]. When zinc deficiency was very mild (5.0 mg Zn intake during the zinc-restricted period), the plasma zinc concentration remained more or less within the normal range and it decreased only after 4-5 mo of zinc restriction. On the other hand, zinc concentrations in lymphocytes, granulocytes, and platelets decreased within 8-12 wk, suggesting that the assay of cellular zinc provided a more sensitive criterion for diagnosing mild deficiency of zinc [30].

We assayed serum thymulin activity in mildly zinc-deficient human subjects [40]. Thymulin is a thymus-specific hormone and it requires the presence of zinc for its biological activity to be expressed. Thymulin binds to high-affinity receptors on T cells, induces several T-cell markers, and promotes T-cell function, including allogenic cytotoxicity, suppressor functions, and interleukin-2 (IL-2) production.

As a result of mild deficiency of zinc, the activity of thymulin in serum was significantly decreased and was corrected by both in vivo and in vitro zinc supplementation. The in vitro supplementation studies indicated that the inactive thymulin peptide was present in the serum in zinc-deficient subjects and was activated by addition of zinc. The assay of serum thymulin activity with or without zinc addition in vitro thus

may be used as a sensitive criterion for the diagnosis of mild zinc deficiency in humans (see chapter 10 for further details)

3.6.4. Cell Culture Studies

Nearly 2000 transcription factors require zinc for their structural integrity; however, it is not known if cellular zinc deficiency results in any change in activation of any of the transcription factors (for further details see chapters 5 and 10). Recent studies have shown several effects of zinc deficiency in cell culture models which have been discussed in chapters 5 and 10.

3.6.5. Role of zinc as an antioxidant and anti-inflammatory agent

Oxidative stress and chronic inflammation are important contributing factors in several chronic diseases, such as atherosclerosis and related vascular diseases, mutagenesis and cancer, neurodegeneration, immunologic disorders, and the aging process. We administered 45mg zinc as gluconate daily to 10 volunteers and 10 subjects received placebo for 8 weeks [45]. The volunteers were healthy and their ages ranged from 19 to 50 years. In subjects receiving zinc, plasma levels of lipid peroxidation products and DNA adduct was decreased, whereas no change was observed in the placebo group. LPS-stimulated MNC isolated from zinc supplemented groups showed reduced mRNA for TNF- α and IL-1 β compared to placebo. Ex vivo, zinc protected MNC from TNF- α induced NF- κ B activation. In parallel studies using HL-60, a promyelocytic leukemia cell line, we observed that zinc enhanced the upregulation of mRNA and DNA-specific binding for A-20, a transactivating factor which inhibits the activation of NF- κ B. Our results suggest that zinc supplementation may lead to down regulation of the inflammatory cytokines through upregulation of the negative feedback loop A-20 to inhibit induced NF- κ B activation [45] (for further details see chapter 4, 10 and 16).

Zinc deficiency, cell-mediated immune dysfunction, susceptibility to infections, and increased oxidative stress has been observed in elderly subjects (> 55 yr old). We conducted a randomized, double-blind, placebo-controlled trial of zinc supplementation in elderly subjects ages 55-87 y [46]. Fifty healthy elderly subjects were recruited for this study. The supplementation was continued for 12 months. The zinc supplemented group received zinc gluconate (45mg elemental zinc) orally daily. Compared with a group of younger adults, at baseline the older subjects had significantly lower plasma zinc, higher plasma oxidative stress markers and endothelial cell adhesion molecules. The incidence of infections and ex vivo generation of TNF- α and plasma oxidative stress markers were significantly lower in the zinc supplemented group than in the placebo group. Plasma zinc and PHA-induced IL-2 mRNA in isolated PMNC were significantly higher in the zinc supplemented group than in the placebo group [46].

In another study, we conducted a randomized, double-blind, placebo trial of zinc supplementation in 40 elderly subjects (age's 56-83 y) and randomly assigned them to two groups [47]. One group received 45 mg elemental zinc daily as gluconate for 6 mo and the other group received placebo. Cell culture studies were also done in order to study the mechanism of zinc action as an atheroprotective agent.

After zinc supplementation plasma high-sensitive C-reactive protein (hs CRP), interleukin-6, macrophage-chemo attractant protein 1 (McP-1), VCAM-1, secretory phospholipase A2, and MDA+ HAE decreased in the elderly subjects in comparison to the placebo group [47]. In cell culture studies, we showed that zinc decreased the generation of TNF- α , IL-1 β , VCAM-1, and MDA+HAE and the activation of NF- κ B and increased

A-20 and peroxisome proliferator-activated receptor- α in human monocytic leukemia THP-1 cells and human aortic endothelial cells compared to the zinc deficient cells. These data suggest that zinc may have a protective effect in atherosclerosis because of its anti-inflammatory and antioxidant functions [47] (for further details see chapter 17).

3.7. Zinc Deficiency and Cognitive Impairment

Penland et al. [48] conducted a study in elementary schools in low-income districts of three provinces in China, in order to assess the effects of zinc supplementation on growth and neuropsychological functions of children. Three hundred-seventy-two children between the ages of 6 to 9 year old were recruited for this study. Supplementation were 20 mg zinc, 20 mg zinc with micronutrients, or micronutrients alone. The micronutrient mixture was based on guidelines of the US NAS/NRC.

Growth was assessed by the change in length of the lower leg. Neuropsychological functions were tested by using the cognition psychomotor assessment system revised (CPAS-R) developed by Penland. Zinc alone had the least effect on growth, while zinc with micronutrients had the largest effect; micronutrients alone had an intermediate effect. Zinc containing treatments improved neuropsychological functions but micronutrients alone had little effect. Performance after zinc alone and/or zinc with micronutrients was better than after micronutrients alone for continuous performance, perception (matching of complex shapes), visual memory (delayed matching of complex shapes), tracking of a cursor on the computer screen, concept formation (identification of oddity) and key tapping.

It is evident that the Chinese children were deficient also in other nutrients besides zinc inasmuch as the group receiving micronutrients with zinc showed the maximum growth. These results are similar to the report published from Iran, which showed that repletion of latent other micronutrient deficiencies was essential for demonstration of the effects of zinc supplementation on growth (for further details see chapters 18-20).

4. Diagnostic Criteria for Zinc Deficiency

Measurement of zinc level in plasma is very useful provided the sample is not hemolyzed or contaminated. In patients with acute stress or infection, or following a myocardial infarction, zinc from the plasma compartment may redistribute to other tissues, thus making an assessment of zinc status in the body difficult. Intravascular hemolysis would also increase the plasma zinc level inasmuch as the zinc in the red cells is much higher than in the plasma.

Zinc in the red cells and hair may be used for assessment of body zinc status. However, inasmuch as these tissues turn over zinc slowly, their zinc levels do not reflect recent changes with respect to body zinc stores. Zinc determination in granulocytes and lymphocytes, however, reflect the body zinc status more accurately and is thus a useful measurement [30]. A quantitative assay of alkaline phosphatase activity in the granulocytes is also a useful tool in our experience [30].

Urinary excretion of zinc is decreased as a result of zinc deficiency. Thus, determination of zinc in 24-hour urine may be of additional help in diagnosing zinc deficiency provided cirrhosis of the liver, sickle cell disease, chronic renal disease, and other conditions known to cause hyperzincuria are ruled out. Hyperzincuria may be associated with zinc deficiency in the above mentioned diseases.

In our volunteer experiments, during the zinc-deficient state our subjects showed a marked positive balance for zinc [49]. Thus, a metabolic balance study may clearly

distinguish zinc deficient from zinc-sufficient state. One may also suggest that perhaps a test based on oral challenge of zinc and subsequent plasma zinc determination may be able to distinguish between zinc deficient and zinc sufficient states in humans.

Kaji et al. [50] measured zinc clearance following I.V. injections of zinc sulfate solution ($1 \mu\text{ mol/kg}$) in Japanese children with low stature. The body zinc clearance test was much more useful than serum zinc concentration in diagnosing marginal zinc deficiency according to these investigators.

We assessed the efficiency of zinc absorption as well as endogenous zinc excretion during a 6-month period of dietary zinc restriction in human volunteers by using a stable zinc isotope (^{70}Zn). Efficiency of zinc absorption was not sustained when the zinc-restricted diet was continued for 6 months [49]. We also observed a decrease in urinary zinc excretion as a result of zinc restricted diet. Our studies indicated that measurement of endogenous intestinal zinc excretion and urinary excretion, both of which are decreased, may be useful for diagnosing marginal deficiency of zinc in humans.

In our studies in experimental human model we observed that a decrease in serum thymulin activity, decreased production of IL-2, decrease in lymphocyte ecto-5' nucleotidase activity, decrease in intestinal endogenous zinc excretion and decrease in urinary zinc excretion occurred within eight weeks of the institution of a zinc restricted diet (approximately 5 mg zinc daily intake) [30]. These changes were observed prior to the changes in plasma zinc concentration and changes in lymphocyte and granulocyte zinc concentration. The decrease in plasma zinc was observed at the end of twenty weeks and decrease in zinc concentration of lymphocyte and granulocytes were observed at the end of twelve weeks of zinc-restricted diet.

The activities of many zinc-dependent enzymes have been shown to be affected adversely in zinc-deficient tissues. Three enzymes, alkaline phosphatase, carboxypeptidase, and thymidine kinase, appear to be most sensitive to zinc restriction in that their activities are affected adversely within 3-6 days of institution of a zinc-deficient diet to experimental animals. In human studies, the activity of deoxythymidine kinase in proliferating skin collagen and alkaline phosphatase activity in granulocytes were shown to be sensitive to dietary zinc intake. As a practical test, quantitative measurement of alkaline phosphatase activity in granulocytes may be a very useful adjunct to granulocyte zinc level determination in order to assess the body zinc status in man. Following supplementation with zinc to deficient subjects, a prompt response in the activities of these sensitive enzymes was observed [30].

We reported that a decrease in plasma thymulin activity in zinc deficient subjects was corrected by in vitro addition of zinc to the plasma and our recent data show that decreased IL-2 mRNA in PHA stimulated mononuclear cells by RT-PCR is also corrected by in vitro addition of zinc [51]. These tests, therefore, may be the most definitive diagnostic tools for marginal zinc deficiency in humans. To learn more about state of the art measurement of zinc, please refer to chapter 9.

5. Therapeutic Impact of Zinc

5.1. Zinc as a growth factor

Zinc deficiency is prevalent worldwide and the current estimate is that nearly 2 billion subjects in the developing world may have zinc deficiency. In growing children, this

results in growth retardation and gonadal failure. Intercurrent infections due to immune disorders cause early death, in zinc deficient subjects. Cognitive impairment is another serious consequence of zinc deficiency in humans. All these serious health problems resulting from zinc deficiency have been now known for nearly fifty years. It is indeed very surprising and somewhat disappointing that major health agencies such as WHO, FAO and UNESCO have ignored this major problem for years. However, some supplementation studies were started by these agencies recently.

5.2. Acute Diarrhea in Infants and Children

Among children in developing countries, diarrhea of prolonged duration is an important cause of growth retardation and death [52]. Episodes of diarrhea, which usually resolve within a few days in healthy children, persist longer in children with malnutrition and impaired cellular immunity. In children with severe zinc deficiency, diarrhea is a common manifestation, which responds promptly to zinc supplementation. Diarrhea also leads to excessive loss of zinc and thus sets up a vicious cycle.

A double-blind, randomized, controlled trial of zinc supplementation (20 mg elemental zinc) involving 937 children, 6 to 35 months of age was conducted in New Delhi, India. All the children also received oral rehydration therapy and vitamin supplements. Among the children who received zinc, there was a 23 per cent reduction in the risk of continued diarrhea. When zinc supplementation was initiated within three days of the onset of diarrhea, there was a 39 per cent reduction in the proportion of episodes lasting more than seven days. In the zinc supplemented group there was a decrease of 39 per cent in the mean number of watery stools per day and a decrease of 21 per cent in the number of days with watery diarrhea. The reductions in the duration and severity of diarrhea were greater in children with stunted growth than in those with normal growth. The authors concluded that zinc supplementation resulted in clinically important reductions in the duration and severity of diarrhea in infants and young children [52]. Similar studies have now been done in many developing countries and the results are strikingly similar.

Possible mechanisms of beneficial effect on diarrhea in children include improved absorption of water and electrolytes by intestines, regeneration of gut epithelium or the restoration of its functions, increased levels of enterocyte brush-border enzymes, and enhanced immunologic mechanisms for the clearance of infections, including cellular immunity and higher levels of secretory antibodies (for further details see chapter 11).

5.3. Zinc for the Treatment of Common Cold

The common cold is one of the most frequently occurring human illnesses in the world [53]. More than 200 viruses may cause common cold. These include rhinoviruses (the commonest cause), coronaviruses, adenoviruses, respiratory syncytial virus and parainfluenza viruses. Adults in USA develop an average of two to four colds and children develop an average of six to eight colds per year. The morbidity resulting from this disease and the subsequent financial loss in terms of working hours are substantial. Previously prescribed treatments have not succeeded in providing a consistent or well-documented relief of symptoms.

Eby et al. [54] were the first to test zinc gluconate lozenges in a double-blind, placebo controlled trial for treatment of cold. One 23 mg zinc lozenges or matched placebo was dissolved in the mouth every 2 wakeful hours or after an initial double dose. After 7 days,

86% of 37 zinc treated subjects were asymptomatic, compared with only 46% of 28 placebo-treated subjects who became asymptomatic ($p=0.0005$). Side effects included objectionable taste and mouth irritation.

In order to test the efficacy of zinc acetate lozenges in reducing the duration of symptoms of the common cold, we carried out a randomized, double-blind placebo-controlled trial in 50 ambulatory volunteers recruited within 24 h of developing symptoms of the common cold [55]. Participants took one lozenge containing 12.8 mg of zinc (as acetate) or placebo every 2 to 3 h while awake as soon as they developed cold symptoms. Subjective symptom scores for sore throat, nasal discharge, nasal conjunction, sneezing, cough, scratchy throat, hoarseness, muscle ache, fever and headache were recorded daily for 12 days. Plasma zinc and pro-inflammatory cytokines were measured on day 1 and after participants were well.

Forty-eight participants completed the study (25 in the zinc group and 23 in the placebo group). Compared with the placebo group, the zinc group had shorter mean overall duration of cold symptoms (4.5 vs 8.1 days), cough (3.1 vs 6.3 days) and nasal discharge (4.1 vs 5.8 days) and decreased total severity scores for all symptoms ($p<.002$). Mean changes in soluble interleukin-1 receptor antagonist level differed non-significantly between the zinc group and the placebo group (difference between changes – 89.4 pg/ml).

Administration of zinc lozenges was associated with reduced duration and severity of cold symptoms, especially cough. Improvement in clinical symptoms with zinc treatment may be related to a decrease in pro-inflammatory cytokine levels; however, in this study the observed differences between changes in cytokine levels in zinc and placebo recipients were not significant statistically.

The effect of zinc lozenges on the duration or severity of common cold symptoms has been examined in at least 14 different studies since 1984. Results of trials in which no effect of zinc was demonstrated were criticized for having inadequate sample sizes or for using inadequate doses of zinc or formulations that reduced the release of zinc ions from the lozenges. Zinc acetate and gluconate are suitable salts, inasmuch as zinc ions are released at physiological pH [56]. Several zinc lozenges use glycine or citrate as ligands which prevent release of zinc ions and therefore, are not effective in curing common cold. For further details see chapter 11.

5.4. Zinc Therapy for Wilson's Disease

Wilson's disease is an inherited autosomal disorder of copper accumulation. The excretion of liver copper in the bile is defective and this leads to a failure of excretion of excess copper in the stool. Eventually excess copper accumulates in the liver and brain and damages these vital organs. Patients typically present in the second to the fourth decades of life with liver disease, a neurological disease of the movement disorder type, or a wide array of psychiatric disturbances. In many cases the diagnosis is either missed or delayed.

The clinical presentation may be that of liver, neurological or psychiatric disease [57, 58]. One third of the patients present with hepatitis or chronic liver cirrhosis. If the hepatic failure is rapidly progressive and fulminant, only hepatic transplantation will save the patient. Approximately one-third of the patients present initially with neurological signs and symptoms. After the hepatic store of copper is exceeded, accumulation of copper in brain takes place. The areas of brain, which are most sensitive to copper accumulation, are those that control and coordinate movement. The symptoms include abnormalities of speech, difficulty in swallowing, and abnormalities of coordination of hand and limb

movement and eventually abnormalities of gait and posture are observed. Tremor is a very prominent clinical manifestation. One-third of the patients present with psychiatric disturbances prior to the onset of neurological manifestations. The younger patients (pre-teenage) usually present with hepatic failure.

The gene for Wilson's disease has been now identified and sequenced. This gene codes for a membrane-bound, copper-binding adenosine triphosphatase type protein, which probably acts as a copper pump, in either the plasma membrane or the intracellular membrane. A large number of mutations in this gene causing Wilson's disease have been identified. This complicates the development of an easy DNA test for the diagnosis of Wilson's disease.

It is important to establish diagnosis of Wilson's disease as early as possible since effective therapeutic measures may completely prevent serious damage to vital organs such as the liver and the brain. Assay of blood ceruloplasmin level is helpful, inasmuch as 90% of the patients with Wilson's disease have low levels. A better diagnostic test is measurement of 24 h urinary copper, which is always elevated in patients with Wilson's disease who are symptomatic. Urinary copper, however, may be elevated in patients with obstructive liver disease who do not have Wilson's disease. In the plasma, non-ceruloplasmin bound copper is markedly elevated.

A slit lamp examination for corneal copper deposits (Kayser-Fleischer rings) is a very useful non-invasive diagnostic procedure. This is, however, positive in only 50% of the patients who present with liver disease. In the future, it may be possible to develop a direct DNA test that could be used for screening of most of the mutations of Wilson's disease gene.

The objective of initial treatment is to bring copper levels down, or otherwise affect copper such that new copper toxicity is no longer occurring. It is also desirable to prevent copper from shifting from one pool to the other during the process of initial copper control. Initial copper control treatment may last for 2 to 4 months. The objective of maintenance therapy is to reduce copper burden and increase the margin of safety, and to prevent reaccumulation of copper.

The anti-copper drugs used for the treatment of Wilson's disease include penicillamine, trientine, zinc and tetrathiomolybdate. Penicillamine has been used for a long period of time. It acts by chelating copper. It is an aggressive anti-copper drug, and produces a large initial negative copper balance. The urinary excretion of copper, however, decreases as the excess mobilizable copper pool shrinks. Penicillamine, however, is very toxic.

Several years ago, we were using therapeutic levels of zinc (150 mg elemental zinc orally daily) for the treatment of sickle cell disease (SCD) patients [30]. Our hypothesis was that zinc would act as an anti-sickling agent. We discovered that in therapeutic amounts, zinc induced copper deficiency in SCD patients. This led Brewer et al. to develop zinc as an effective therapeutic modality for the treatment of Wilson's disease [57, 58].

Zinc acts by induction of intestinal cell metallothionein. Metallothionein, once induced, has a high affinity for copper, and prevents the serosal transfer of copper into the blood. The intestinal cells turn over rapidly and take the complexed copper into the stool, where it is excreted. Zinc not only blocks food copper but also the copper, which is endogenously excreted via salivary, gastric, and other gastrointestinal juices. As a result, zinc produces a chronic negative copper balance [57, 58]. Zinc is administered orally. The dosage is 50 mg elemental zinc (as acetate) three times a day, given in a fasting or post-absorptive state. Zinc is a non-toxic drug. The only side effect is that

10% of patients may have gastric discomfort. This is usually confined to the first morning dose, particularly if it is taken before breakfast [57, 58]. This can be mitigated by taking the first dose of zinc between breakfast and lunch.

For maintenance therapy zinc is the treatment of choice [57, 58]. The big advantage of zinc over other drugs is that it has practically speaking no toxic effects. Zinc is also the drug of choice for treatment of pre-symptomatic patients and pregnant women. Whereas, penicillamine and trentine are teratogenic, zinc has no teratogenic effects (for further details see chapter 24).

5.5. Prevention of Blindness in Age Related Macular Degeneration (AMD) by Zinc

Age-related Eye Disease Study group, supported by National Eye Institute, NIH, conducted an 11-center double-masked clinical trial in patients with AMD [59]. 3640 participants were enrolled. Their ages ranged from 55-80 years and the average follow-up period was 6.3 years. Participants were randomly assigned to receive daily oral tablets containing one of the following: 1) Antioxidants (vitamin C 500 mg; vitamin E, 400 IU; and beta carotene 15 mg), 2) Zinc, 80 mg as zinc oxide and copper 2 mg as cupric oxide, 3) Antioxidants plus zinc, or 4) Placebo. Copper was added to prevent copper deficiency in the zinc supplemented group.

Group taking the antioxidant plus zinc supplements reduced the risk of developing advanced AMD by about 25 percent and reduced the risk of vision loss by about 19 percent. Group taking zinc alone reduced the risk of developing advanced AMD by about 21 percent and vision loss by about 11 percent, whereas the group taking the vitamins alone reduced their risks for developing advanced AMD by about 17 percent and vision loss by about 10 percent. No side effects were noted due to therapeutic levels of zinc supplementation. In therapeutic dosage, zinc is an effective *in vivo* antioxidant. Another interesting observation was that only the zinc supplemented group showed increased longevity [60]. The risk of mortality was reduced by 27% in participants of the Age related eye disease (AREDS) aged (55-81y) who received high dose of zinc (80 mg/d) as oxide during median follow up of 6.5 years [60] (for further details see chapter 27).

6. Toxicity of Zinc

Acute high-level exposure to zinc compounds can produce respiratory and gastrointestinal toxicity. However, these effects are largely self-limiting and require only modest medical attention. Exposure to smoke from bombs containing zinc compounds can cause illness, generally transient, as can acute or chronic exposure to fumes from acetylene or electric welding of zinc-containing metals. Exposure to excess zinc tablets intended for human consumption, controllable for the most part can also result in clinical changes, such as decreased activity of erythrocyte superoxide dismutase and increased activity of serum alkaline phosphatase and pancreatic enzymes. Other reported effects such as changes in serum lipoproteins and immunological status are controversial and require more definitive assessment. There is no compelling evidence supporting a cause for concern about zinc in the environment as a putative toxic agent [61]. Ingestion of elemental zinc in excess of 50 mg daily for more than 12 weeks causes copper deficiency [30]. This is manifested by hypochromic microcytic

anemia and neutropenia, which are easily corrected by administration of 2 mg copper daily.

One schizophrenic patient swallowed a Kg of pennies, which led to zinc toxicity and subsequently copper deficiency. Pennies coined after 1982 are made of zinc with copper coating. In this case, sideroblastic anemia and neutropenia were recorded. Sideroblastic anemia is characterized by the accumulation of ferric iron in mitochondria of erythrocyte precursors (normoblasts). The mechanism of this effect is unknown. One hypothesis is that the excess intake of zinc led to copper deficiency. The pathogenesis of this effect needs to be studied, which may facilitate understanding of mechanisms of sideroblastic anemias and possibly management strategies, both of which are lacking at present [62].

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3. Nutritional Aspects of Zinc Consumption

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Abstract. Zinc is most bioavailable from animal flesh, and red meat is the richest common source. In contrast, foods prepared from cereals, legumes, other seeds, and other plant parts may be rich in indigestible zinc-binding ligands, such as phytate and certain dietary fibers, that suppress zinc bioavailability. Zinc absorption from zinc supplements is modulated over time so that the amount of zinc absorbed does not exceed need. Supplements of calcium, iron and folate are reported to suppress zinc absorption under some circumstances. Treatment of zinc deficiency with zinc is most efficacious when other micronutrient are also available in adequate amounts.

Key words, zinc bioavailability, meat, phytate, dietary fiber, Maillard browning

Introduction

Economic status, availability of food, personal choices, food chemistry and host chemistry influence zinc nutriture. Animal flesh is the best source of dietary zinc for humans and red meat is the richest common source. In contrast, many foods derived from plants are low in zinc and/or are rich in indigestible zinc binding ligands. Paleolithic humans were well adapted to diets of animal flesh supplemented with fruit, leafy and root vegetables, as are modern humans. Transformation of the human food supply to a limited dependence on animal flesh and increased dependence on cereals and legumes rich in indigestible zinc binding ligands such as phytate and certain dietary fibers, changed the dietary dynamic so that increased risk of zinc deficiency became endemic among all but the most affluent humans. As pointed out by Solomons [1] “At no time during the last 400 generations, i.e., throughout the agricultural era, has either the intake of zinc or its bioavailability been as high as it was for the 10,000 generations that preceded it.”

1. Diet Zinc

1.1. Zinc Content of Foods

The zinc content of foods varies widely (**Table 1**). According to Solomons [1] about half of all foods provide < 1 mg zinc / 100 g edible portion: included are “beverages,

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milk and dairy products, fin-fish, cooked breakfast cereals, such as oatmeal, cream of wheat and farina, refined grain products, tubers, vegetables, fruits, soups, nuts, shortenings and oils, spreads, spices, sweeteners and syrups, and sweets and miscellaneous candies.” In contrast, most “meats, nuts, seeds, legumes, and whole-grain items” provide > 1 mg zinc / 100 g edible portion. Meat has been reported to provide nearly 50 % of the zinc in US diets while dairy products provide about 20 %, and most of the remainder is provided by cereals and legumes [2]. In contrast, lacto-ovo-vegetarian diets provide zinc from cereals (about 16 %), legumes and nuts (about 26 %), and milk and eggs (about 18 %).

Table 1. Zinc in representative foods in the United States, per common measure [3, 4].: * Foods rich in zinc binding ligands.

> 15 mg	5-10 mg	4-5 mg	3-4 mg	2-3 mg	1-2 mg	< 1 mg
Oyster	Beef	Beef liver	Lamb	Lamb	Pork loin	Chicken breast
Peanut Butter *	Lamb	Beef	Pork	Pork	Chicken dark meat	Chicken liver
Product 19 ® *	Duck	Lamb	Veal	Lobster	Sword fish	Salmon
Total ® *	King Crab	Pork	Turkey dark meat	Clam	Shrimp	Tuna
Wheaties ® *	Captian Crunch ® *	Blue Crab	Yogurt	Mushroom	Other Finfish	
Quaker ® *	Oats	Rice Chex ® *	Skim Milk	White wheat flour	Vegetables	
		Corn Chex ® *	White bean *	Navy bean *	White rice	
		Cheerios ® *	Chick pea *	Black bean *	Egg	
		Whole wheat flour *	Lentil *	Pinto bean *	Tofu	
		Corn meal *	All Bran ® *	Cheddar cheese		
			Nuts *	Blue cheese		
				Cottage cheese		

1.2. Dietary Zinc and the “Estimated Average Requirement”

Representative median zinc intakes [5] and the “estimated average requirement” (EAR) published in the Dietary Reference Intake of the Institute of Medicine, USA [6] are listed in **Table 2**. The EAR assumes the diet is free of inhibitors of zinc absorption. Given that half of the population requires more zinc than the EAR, it appears that proportion of women and elderly at risk of zinc deficiency is greater than among men. For them food selection is especially important. It must be noted however that the mere presence of plentiful zinc is not necessarily an indicator of zinc adequacy. For example, diets of farmers in Iran among whom zinc deficiency was first recognized, contained plenty zinc. However the high phytate and dietary fiber content suppressed

zinc absorption to such a degree that zinc deficiency was likely, especially in individuals that practiced geophagia [7, 8].

When income is not the primary factor affecting zinc nutriture, food choice becomes increasingly important. Thus selection of chicken or fin-fish instead of red meat by elderly increased risk of zinc deficiency **Table 1** [9]. The high importance of food choices that include meat is further illustrated by findings from a case control study by Velie et al [10] of 859 women. They found that preconceptional diets rich in animal protein and zinc,

Table 2. Median zinc (mg/d) consumption [5] of some age (y) groups in the United States and their estimated average (mg/d) requirement (EAR) [6]¹.

Age	White		Hispanic		Black		EAR
	n	Median	n	Median	n	Median	
Males							
3-5	219	7.3	281	7.8	210	8.1	2.5 to 4y:4
12-15	98	11.6	129	10.5	95	8.9	7 to 14y: 8.5
20-29	216	13.1	349	13.3	245	12.9	9.4
60-69	247	11.5	152	8.7	141	8.8	9.4
80+	250	9.1	19	7.7	21	7.0	9.4
Females							
3-5	206	6.5	328	6.8	244	7.5	2.5 to 4y: 4
12-15	123	8.1	140	8.7	96	8.5	7.0 to 14y: 7.3
20-29	244	8.4	317	8.8	254	8.9	6.8
60-69	246	7.7	153	6.8	148	6.9	6.8
80+	251	6.6	23	5.3	35	5.9	6.8

and/or supplemental zinc were associated with an absence of neural tube defects (NTDs), while diets rich in plant protein and phytate were associated with presence of NTDs. Similarly, Scholl et al [11] found that maternal diets with more than 6.1 mg zinc daily were associated with higher fetal weight and lower risk of prematurity.

1.3. Zinc Functions in Concert with Other Nutrients

Zinc is a stable divalent cation with an ionic radius that facilitates coordination with thousands of proteins that require zinc for function. These zinc binding proteins facilitate metabolism in concert with other micronutrients [12], and optimal function requires that all are present in appropriate amounts. Because micronutrients are unevenly distributed among foods [13] consumption of a wide variety is necessary to achieve an adequate intake of all micronutrients. Absent this luxury, risk of micronutrient deficiencies is increased. Thus multiple subclinical (hidden) micronutrient deficiencies are part of the background against which “classical” deficiencies and less well characterized nutritional illnesses occur. Indeed, micronutrient and other deficiencies that are endemic in communities are part of the environmental fabric on which non-nutritional illnesses occur. The consequences of multiple micronutrient deficiencies on clinical response to treatment with single or two micronutrients were measured in children by Allen, et al [14]. They compared the respective efficacy of treatment with a multi-micronutrients, placebo, single or two micronutrients on length, weight, concentrations of hemoglobin, zinc, retinol, and motor development. Multi-micronutrients were the most effective.

As far as zinc is concerned, the phenomenon was first appreciated in zinc deficient village children from Egypt and Iran who were treated with zinc alone for retarded growth retardation and sexual development [15, 16]. Little if any increase in growth

occurred. This was in stark contrast to growth responses of zinc deficient stunted, underdeveloped adolescents who were treated with zinc while being fed an omnivorous diet adequate in other nutrients [17, 18]. Subsequent treatment of the village children with zinc and multi-micronutrients were more effective [19, 20], but less than expected presumably because of high consumption of bread rich in phytate.

A more recent double blind randomized controlled treatment trial of zinc and other micronutrients in about 750 Chinese school children, aged 6-9 years by Sandstead, et al [21, 22] confirmed that zinc was most effective when administered with other micronutrients. Increases of stature, neuropsychological functions and immunity was significantly greater after treatment with zinc and a broad mixture of other micronutrients compared to treatment with other micronutrients alone or zinc alone.

In addition to occurring against a background of subclinical micronutrient deficiencies, zinc deficiency also occurs simultaneously with iron deficiency [23]. This association was evident in the first index cases of zinc deficiency [24]. The possibility that this association might be useful for identifying zinc individuals and groups at high risk of zinc deficiency was shown by Yokoi, et al [25]. They found that premenopausal women not taking oral contraceptives, with serum ferritin concentrations of 20 mg/L or less were highly likely to be zinc deficient based on the size of their 24 hour rapidly exchangeable tissue zinc pool.

1.4. Measurement of Zinc Retention from Diets

The most reliable method for measurement of zinc retention involves use of trace amounts of the γ -emitting isotope ^{65}Zn and physical measurement of ^{65}Zn retention by the whole body. Modern whole body counters (WBC) are highly sensitive and when used correctly are unlikely to produce errors [26-29]. Errors associated with other methods for measuring retention that include specimen collection and chemical analyses are avoided.

The ^{65}Zn , is administered in trace amounts, 3.70 to 7.4 kBq (0.10 to 0.2 mCi). Retention is measured serially by WBC. Radiation exposure is less the range many humans experience during daily living from their intrinsic ^{40}K , environmental radon, high altitude flying, and diagnostic X-rays. After ^{65}Zn is administered its initial disappearance from the body is rapid as the unabsorbed ^{65}Zn is excreted in feces. Then the loss slows, and the retention curve is linear on a semi-logarithmic plot (the natural logarithm of percentage remaining radioactivity versus time). Regression to the Y-axis gives the ^{65}Zn retained (fractional zinc absorbed, FZA). Because the ^{65}Zn tracer distributes in chyme in a manner similar to food zinc, the quantity of zinc absorbed (QZA) can be calculated if the total diet zinc is known. This approach was used by Hunt et al in the illustrative studies that follow.

1.5. Animal Flesh

Animal flesh is the primary source of highly bioavailable zinc for humans. Zinc content of beef, veal and other red meats is substantially greater than zinc content of white meats and fin-fish (**Table 1**). This has implications for the recommendations from some "health authorities" that the replacement of red meat in diets with chicken and/or fin-fish will improve health. To the contrary, such a choice is likely to adversely affect zinc nutriture, as illustrated by a controlled metabolic study by Milne et al [30] in

which zinc deficiency was induced in otherwise healthy men by feeding diets in which white chicken meat and fin-fish meat were the primary animal protein sources.

1.5.1. Retention of Zinc from Diets High or Low in Meat, and Low Meat with Zinc

Hunt et al [31] fed 14 adults 3 omnivorous diets for 7 weeks each in random order and measured their FZA by 3 methods: WBC, the difference between ^{65}Zn in diet and feces, and chemical balance. Three 2-day menus provided: 289 g/d of beef, pork, poultry and fish, in proportions usual in USA diets; 38.5 g/d of meat, with energy from meat protein replaced by low-mineral sources of carbohydrate (fruits and sugars) and animal fat replaced by vegetable fat; or the 38.5 g/d of meat diet enriched with potassium, phosphate iron, magnesium and zinc (as gluconate) so that totals were similar to the high meat diet. All diets provided about 13.5 g dietary fiber and 1056 mg phytic acid per 2200 kcal. After the diets were fed for 2 weeks, ^{65}Zn was administered in ground-meat, and serial WBC were done for 5 weeks; diet and fecal ^{65}Zn were measured from the day of ^{65}Zn administration to the end of feeding; and zinc balance was measured the last 18 days of the feeding. The FZA was similar during the high and low meat diets (**Table 3**), but QZA was greater from the high meat diet ($p < 0.05$). Thus ability to increase the FZA was apparently limited. The addition of zinc gluconate to the low meat diet did not increase the QZA. As expected, methods other than WBC gave lower FZA and QZA.

Table 3. Mean fractional zinc absorbed (FZA) and quantity (mg) zinc absorbed (QZA) from diet 1) high meat, 2) low meat, and 3) low meat diet supplemented with zinc gluconate; measured by WBC, ^{65}Zn tracer in feces/diet, and chemical balance [31].

Diet Zn mg		WBC		^{65}Zn in feces/diet		Balance	
		FZA	QZA	FZA	QZA	FZA	QZA
1) 13.0	14	0.28	3.6	0.21	2.7	0.16	2.1
2) 6.7	14	0.30	2.0	0.22	1.5	0.03	0.2
3) 11.6	14	0.18	2.1	0.12	1.4	0.08	0.9

1.6. Plant-Based Foods

Plant-based foods provided by agriculture became the nutritional base for post Paleolithic society. As agriculture evolved more food became available and associated populations grew. Development of technologies for processing plants must have begun before agriculture. Today technology makes a wide variety of products possible including the fortification of foods with microencapsulated micronutrients. However the availability of such technology is limited. Traditional fermented foods of some cultures offer improved bioavailability of zinc and other essential metals that are bound to phytate and dietary fibers. In spite of advances, low bioavailability of zinc and other essential metals from traditional whole grain unfermented flat breads continues to be a problem for many populations.

1.6.1 Intestinal Contents Affect Zinc Absorption

Amino acids and peptides in chyme facilitate zinc absorption, while indigestible zinc binding ligands from plants, such as phytate and dietary fibers inhibit zinc absorption. Gastric acid and pepsin facilitate the solubility of zinc bound to peptides and other

ligands in the stomach and proximal duodenum. As chyme moves distally and pH increases zinc binds with indigestible ligands. However zinc absorption is not completely suppressed; zinc secreted into chyme in the upper small intestine is partially recovered by the distal small intestine and cecum [32, 33]. Humans do not make enzymes that hydrolyze phytate and certain dietary fibers, but certain colonic bacteria do produce phytase. In contrast, there is little if any hydrolysis of Maillard browning products.

1.6.2. Phytate

Human dependence on foods rich in phytate is a major cause of the high prevalence of zinc deficiency worldwide. Phytate is the anionic form of the cyclic sugar alcohol myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate (IP6) present in mature seeds. It forms stable complexes with di- and trivalent cations such as Zn²⁺, Cu²⁺, Ni²⁺, Co²⁺, Mn²⁺, Fe²⁺/Fe³⁺, and Ca²⁺ [34]. Phytic acid has 12 replaceable protons with 6 having a pK of 1.5-2.0, 2 of 6.0, and 4 of 9.0-11.0. Thus in gastric chyme zinc tends to be in solution, but at higher pH in distal duodenum and beyond zinc is likely to complex with phytate. And, when two cations are present simultaneously the proportion of IP6-metal complex increases. Hydrolysis of IP6 by phytase is inhibited by IP6-metal complex.

The practical importance of phytate for humans became evident when Harrison and Mellanby [35, 36] showed that oatmeal rich in phytate caused calcium deficiency and rickets in young dogs. Related findings by McCance and Widdowson [37, 38] showed that bread prepared from 92 % extraction wheat flour rich in phytate suppressed calcium retention of humans. The importance of binding of phytate for zinc nutriture was evident from studies in other species [39-41]. Later phytate was shown to suppress zinc absorption of humans and thus contribute to zinc deficiency [7, 8, 42]. In addition, it was evident that humans are unable to adapt to phytate [43].

Aware of the adverse effects of phytate on human zinc absorption, Solomons, et al [44] measured effects of maize tortilla (a flat bread prepared from lime treated maize, and cooked on a very hot surface) and frijoles (re-cooked red beans) on absorption of zinc, using oyster as the source of zinc. The rise in plasma zinc after a meal of 120 g of oysters was compared to the increase after the same amount of oysters eaten with 120 g of either frijoles or tortilla. Frijoles suppressed the increase in plasma zinc about 50 %, while the suppression after tortilla was nearly 100 %. Thus, the reach of ancient cereal grain ethnic foods in the pathogenesis of zinc deficiency was confirmed to extend to the Americas.

The high importance of dietary phytate as a cause of zinc deficiency was further shown by Hambidge, et al [45]. Using a trivariate model of the quantity of zinc absorbed as a function of dietary zinc and phytate with updated parameters they estimated the effect of phytate on zinc needs of men and women. They found the EAR predicted by the model at 0 phytate was similar to the EAR of the IOM [6]. Addition of 1000 mg phytate doubled the EAR and 2000 mg phytate tripled the EAR. Thus according to the model, when the total dietary phytate is 1000 mg/d, women require about 13 mg of dietary zinc and men require about 18 mg of dietary zinc. In addition, the EAR for men and women is unlikely to be attained when phytate:Zn molar ratios were 11 to 1 and 15 to 1, respectively.

1.6.2.1. Effect of Phytate, and the Quantity of Zinc on Zinc Absorption

Humans are unable to adapt to habitual consumption of phytate [43]. Hunt et al [46] confirmed this phenomenon and also measured zinc absorption relative to zinc needs. The 109 subjects, aged 21-51 years, who were fed controlled diets, for 4 or 8 weeks, while living at home. Subjects were randomly assigned 1 of 10 diets with zinc contents ranging from 4 to 29 mg daily. Five diets had phytate:zinc molar ratios of 2 to 7, and 5 had phytate:zinc molar ratios of 15 to 23. Zinc retention was measured by 4 weeks of serial WBC of ^{65}Zn (7.4 kBq) that was administered twice, at baseline, and at the end of the 4th week. When 39 subjects divided into groups of 8 were fed one of the 5 low phytate diets for 4 weeks (**Figure 1**), the mean FZA at baseline (0.24-0.49) and follow-up (0.23-0.70) were inversely related to diet zinc, and follow-up exceeded baseline ($p = 0.01$).

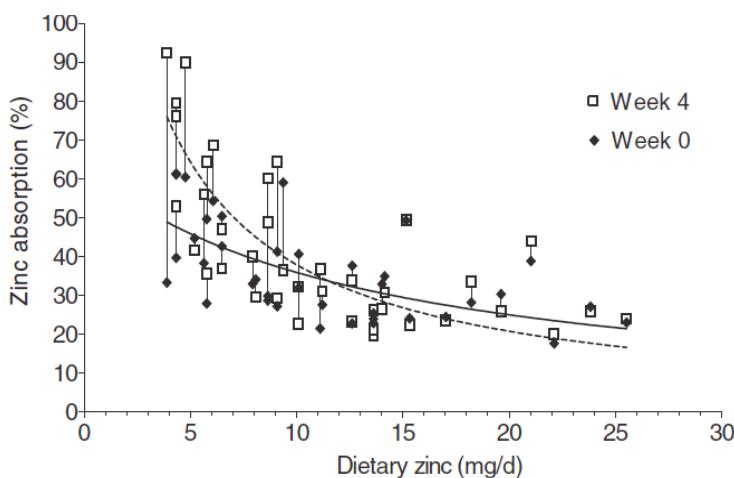


Figure 1. Zinc retention relative to quantity of dietary zinc (72). Thirty-nine subjects were fed specific constant amounts of zinc from baseline through 4 weeks. Zinc retention from the 2-day constant menu was measured at baseline and 4 weeks by whole body counting (WBC) of ^{65}Zn tracer fed in the 2-day menu at baseline and at 4 weeks. Baseline fractional zinc absorption (FZA), expressed by the solid line, and FZA after consumption of the diet for 4 weeks, expressed by the broken line cross at about 12 mg dietary zinc. However, it appears the higher follow-up zinc absorption (open box) compared to baseline (closed diamond) stopped when diet zinc was about 9 mg daily. FZA was inversely related to diet zinc: $\text{FZA} = \frac{A_{\max}}{(K_R + TDZ)}$, $n = 39$, $R^2 = 0.40$ at baseline and $R^2 = 0.65$ after 4 weeks (A_{\max} , maximum absorption; K_R , relative binding constant between zinc and transport receptors; TDZ, total dietary zinc).

At the lowest concentration of diet zinc FZA increased to > 0.90 . The lines representing the average pre and post FZA crossed when dietary zinc was about 12 mg. Not shown, the mean QZA at baseline (2.4-4.2 mg) and follow-up (3.3-5.0 mg) were directly related to diet zinc, and follow-up exceeded baseline ($p = 0.06$). A second group of 26 subjects who were studied for 8 weeks confirmed the inverse relation of FZA to diet zinc and the increase in FZA and QZA over time ($p = 0.0005$ and 0.005). High phytate (and other zinc binding ligands) decreased the FZA and QZA at baseline and follow-up. Baseline FZA (0.15-0.39) was inversely related to diet zinc, and was unchanged at the 4 week follow-up. The baseline QZA levels (2.4-3.1 mg) were similar to the 4 week follow-up. Modeling by Miller et al [47] suggests that diet phytate:zinc molar ratio ≥ 12 substantially increases the risk of zinc deficiency.

1.6.3 Other Dietary Inhibitors of Zinc Retention

Findings of Reinhold et al [48-52] indicate that zinc absorption can also be inhibited by dietary fibers, cellulose, dextrans, and Maillard browning products. In addition, Kelsay et al [53] reported that fruit and vegetable fiber inhibit zinc absorption, but did not iron absorption [54], perhaps because of the presence of ascorbic acid [55].

Various foods and other factors apparently affect risk of zinc deficiency in young women [25] In addition to a highly significant correlations with serum ferritin concentration and the quantity of menstruation, the size of the rapidly exchangeable "tissue" zinc pool in a 3-compartment model correlated significantly with the frequency of consumption of certain foods. In 30 subjects the correlation with food frequency, $R^2 = 0.545$, $p = 0.002$, included 6 foods: yoghurt ($r = 0.525$, $p = 0.008$), coffee ($r = 0.507$, $p = 0.011$), beef ($r = 0.467$, $p = 0.021$), bran breakfast cereal ($r = -0.611$, $p = 0.002$), orange juice ($r = -0.451$, $p = 0.027$), and eggs ($r = -0.350$, $p = 0.094$). The positive association of beef and negative association of bran cereal were expected. Perhaps the associations of yoghurt and coffee reflected acidity; the association of orange juice reflected fruit fiber; and the association of eggs reflected phosphate.

1.6.3.1. Maillard Browning Products

Maillard browning products have been present in foods since cooking was invented. In the early 20th century Louis-Camille Maillard described the condensation of sugar carbonyls and aldehydes with amino acids to form brown substances that in the analysis of dietary fiber are found in the crude lignin fraction [56]. They are about 11% nitrogen, and are not digestible by humans or anaerobic bacteria. When Reinhold and Garcia [52] cooked maize masa (dough) on a very hot surface to produce tortilla, the percentage neutral detergent fiber and acid detergent fiber increased significantly because of browning. The effect of browning products on human intestinal absorption of zinc was measured by Lykken et al [57]. They compared absorption of ^{65}Zn incorporated into maize during growth, from maize (corn) flakes and from maize dough. Absorption from the flakes was significantly less than from the dough. Relevant to these findings, Whitelaw and Weaver [58] condensed glycine, D-leucine, L-proline, L-lysine and L-glutamic acid with D-glucose by autoclaving at 110°-120°C, at 15 atm, for 10 min, to produce 6-8 KD non-dialyzable ^{65}Zn binding ligands. In addition, they showed that toasting degerminated maize meal, labeled with ^{65}Zn , at 220°C for 25 min, resulted in ^{65}Zn binding to non-dialyzable ligands. Consistent with these findings, O'Brien and Morrissey [59] showed though potentiometric proton displacement analysis the glucose-glutamate binding of Zn^{2+} , Cu^{2+} , Mg^{2+} and Ca^{2+} . The strength order was: $\text{Mg}^{2+} > \text{Cu}^{2+} = \text{Ca}^{2+} > \text{Zn}^{2+}$.

Birlouez-Aragon et al [60] reported that a diet rich in browning products increased metabolic signs associated with increased risk adult onset diabetes and cardiovascular diseases in healthy people. Some products of Maillard browning appeared in urine. In this context it is of interest that browning products administered intravenously increased loss of zinc [61]. A connection between zinc loss and in vivo adverse of browning products remains to be shown.

1.6.4. Vegetarianism and Zinc Absorption

Hunt et al [62] compared effects of lacto-ovo-vegetarian and omnivorous diets on zinc absorption in 21 women, aged 20-42 years, over a 16 week time period, with a cross over at 8 weeks, while subjects lived at home. The omnivorous diet provided 184 g meat (2/3 beef) daily. Chemical balance was measured in all 21 subjects. In 11 subjects ^{65}Zn labeled food was fed at the beginning of the 5th week of the respective diets and serial WBC measurements were done during the 4 weeks to the end of the diet. The FZA from the omnivorous diet exceeded the FZA from the lacto-ovo-vegetarian diet ($p < 0.01$), while the QZA was 1.3 mg greater ($p < 0.001$) (**Table 4**). In contrast, chemical balance did not detect differences in the FZA or QZA of the two diets.

Table 4. Effects of Omnivorous and Lacto-ovo-vegetarian diets on fractional zinc absorbed (FZA) and quantity (mg) zinc absorbed (QZA) measured by WBC and chemical balance [62]

Diet	Diet Zn mg	Phytic acid mmol	Phytate:Zn molar ratio	WBC		Balance	
				FZA	QZA	FZA	QZA
Omnivorous	11.1	821	5	11	0.33	3.7	21
Lacto-ovo-Veg	9.1	2509	14	11	0.26	2.4	21

In addition, consumption of the lacto-ovo-vegetarian diet was associated with a significant decrease in plasma zinc within the “normal” range, and a decrease in hair zinc. In addition the QZA from lacto-ovo- vegetarian diets was less than the 3.2 mg zinc current knowledge suggests women need to absorb daily [6].

1.7. Adaption of Zinc Absorption from Zinc Supplements

The easy availability of over-the-counter zinc supplements in the US marketplace prompted Beiseigel et al [63] to measure effects of daily zinc supplementation on zinc retention. The subjects, 12 post-menopausal women, consumed controlled diets low in phytate for 22 weeks that provided about 5 mg zinc daily. Supplements were administered to 6, 3 and 3 subjects that provided 14, 32 and 47 mg zinc. Zinc retention was measured 3 times by WBC of ^{65}Zn administered in the 1-day menu at the beginning of the experiment and after 8 and after 16 weeks. The respective QZA at baseline were 4.6, 8.7 and 10.3 mg ($p < 0.05$). These differences disappeared after 8 and 16 weeks; and by 16 weeks the QZA was about 5 mg regardless of supplement level.

These findings are consistent with increased induction of metallothionein synthesis and binding of zinc to metallothionein, resulting in modulation of the quantity of zinc available for transport into the body. The induction of metallothionein also results in binding of copper. The high binding affinity of metallothionein for copper can result in copper deficiency, with injury of the cardiovascular [64-67], nervous [68-70], skeletal [71], connective tissue [72, 73] and the hematopoietic [74] systems. Turnover of enterocytes returns zinc and copper to intestinal chyme.

1.8. Other Nutrients that can Suppress Zinc Absorption

1.8.1. Calcium

The combination of calcium and phytate in experimental animal diets suppressed zinc absorption [41, 75, 76]. In contrast the addition of calcium to human diets reported by

Spencer [77, 78] did not suppress zinc absorption. More recently Wood and Zheng [79] reported that 468 mg calcium from milk or as dibasic calcium phosphate did not suppress zinc absorption from an omnivorous diet. However, when they increased dietary calcium to 1358 mg daily zinc retention was suppressed, and in addition, the found that lavage of the duodenum with 600 mg calcium in solution suppressed zinc absorption, which was partially ameliorated by adding zinc to the lavage [80]. Other studies in young children by Gibson et al [81, 82] showed an interaction of calcium and phytate that suppressed zinc nutriture.

1.8.1.1. Effects of Calcium and Phytate on Zinc Absorption

Hunt et al [83] measured acute effects of phytate and calcium on zinc retention of 10 healthy women, aged 21-50 years, who consumed 2-day experimental diets at the research center at the beginning of four 4-week experiments in random order. On the second day of the experimental diet ^{65}Zn was administered during each of the three meals, added to the richest sources of zinc. Subjects then consumed self selected diets for 4 weeks while zinc retention was measured serial WBC. One day intakes of zinc, phytate and calcium from the diets and the FZA and QZA are shown in **Table 5**.

Table 5. One day intakes of zinc, phytate and calcium, and fractional zinc absorbed (FZA) and quantity of zinc absorbed (QZA) from omnivorous western diets that provided 2200 kcal of energy and 85 g protein [83]

Zinc mg	Phytate mg	Calcium mg	Phy:Zn mols	Phy x Ca:Zn mols	FZA	QZA mg
11.5	483	669	4.2	69	0.33	3.8
11.4	391	1897	3.4	161	0.39	4.5
11.3	1781	697	15.6	272	0.29	3
12.2	1789	1719	14.5	623	0.26	3.2
FZA, p	< 0.001	0.17		0.08		
QZA, p	< 0.001	0.09		0.3		

The 1-day increase in phytate of about 1300 mg suppressed FZA about 25 % and QZA about 1 mg. The 1-day increase in diet calcium, which was accomplished by feeding foods commercially enriched with calcium, had no effect on FZA and QZA.

1.8.1.2. Effects of Iron on Zinc Absorption

Solomons and Jacob [84] used the increase in plasma zinc after oral administration of 25 mg zinc as zinc sulfate or of 54 mg zinc in oyster, as a baseline against which to measure effects of ferrous sulfate on zinc absorption. Proportions of iron to zinc were: 1 to 1, 2 to 1, and 3 to 1. “Slight” inhibition of zinc absorption occurred at a ratio of 1 to 1, while “substantial” inhibition occurred at ratios of 2 to 1 and 3 to 1. In contrast, heme iron chloride had no effect on zinc absorption at a 3 to 1 molar ratio, and ferrous iron at a 2 to 1 ratio relative to zinc in oyster had no effect on zinc absorption. A subsequent study found ferric iron was significantly less inhibitory at a 2 to 1 ratio. Inclusion of ascorbic acid increased the inhibitory effect [85].

1.8.1.3. Effects of Folate on Zinc Absorption

Milne et al who were conducting highly controlled studies of zinc metabolism [30, 86] serendipitously observed [87] that administration of 400 micrograms of pteroylglutamic acid (folic acid) every other day caused negative zinc balance in 8 men fed diets containing 150 micrograms of folacin (by analysis), and about 7.5 mg zinc daily for 4 weeks followed by about 3.5 mg daily for 16 weeks. Fecal zinc increased ($p < 0.001$) and urine zinc decreased about 50%. When 30 mg zinc was administered for 4 weeks the folate effect disappeared. No change occurred in iron or copper balance. These findings prompted an evaluation of effects of folate supplementation as routine pregnancy care on fetal outcomes [88]. Comparison of the lowest and highest quartiles of serum folate concentrations in 394 adult mothers from Ohio found high serum folate associated with more pregnancy complications ($p < 0.008$) and fetal distress ($p < 0.002$) than low serum folate. High serum folate was also associated with significantly increased fetal complications in black teenage mothers from New Orleans [89]. Milne et al [90, 91] subsequently confirmed his earlier studies by measurement of effects of folate on ^{65}Zn absorption using WBC.

Simmer et al [92] reported that folate suppressed zinc absorption, measured by the increase in plasma zinc after oral administration of zinc sulfate, of by 10 non-pregnant adults. These findings were confirmed by Ghishan, et al [93] who showed in rats, both *in vivo* and *in vitro*, that folate decreased zinc transport from the lumen of the intestine, and also that zinc decreased transport of folate from the intestine. More recently, Fuller et al reported serum folate concentrations were inversely related to serum zinc concentrations in 60 preterm infants who had been administered of as much as 1.0 mg folic acid daily.

Notably, the above findings were not confirmed by several investigators. Keating, et al [94] through a series of studies in rats and humans found no relationships between folate administration and zinc nutriture. Tamura et al [95] also found no adverse association between folate administration and zinc status of 285 pregnant mothers, and found improved pregnancy outcomes associated with folate administration. In addition, Kauwell et al [96] found no effects of 0.8 mg folate daily on zinc status of 12 men fed 3.5 mg zinc for 25 days.

2. Conclusion and Perspectives

2.1. Prevention of Zinc Deficiency

Bioavailability of zinc from human diets is a major problem worldwide. Some of the difficulties obstructing prevention are noted above. Their practical solutions are elusive. Because dietary preferences are deeply engrained in culture, it seems that solution of the problem will require an approach that appears culturally neutral. Fortification of common foods with specific micronutrients, without changing the organoleptic qualities of the food has been in part responsible for the near disappearance of beri-beri, pellagra, rickets, goiter and blindness in regions where the method has been effectively applied. Prevention of zinc deficiency will require fortification of selected foods with zinc, and all other micronutrients with which functions in concert. Microencapsulation of selected micronutrients so as to prevent peroxidation and off flavors will facilitate this process. Targeted fortification of a specific food with cultural cachet might be the

approach most likely to succeed. Such an approach might facilitate targeted interventions in groups at high risk.

2.2. Revision of Dietary Recommendations

Dietary recommendations that propose restriction of consumption of red meat and increased consumption of unrefined cereal grains and legumes have the potential to increase risk of zinc deficiency. Because zinc is so important for development of the embryo and fetus [97, 98] risk is especially high among women of childbearing age [10, 25], with implications for their progeny [99, 100], and possibly their grand children [101]. Other groups at increased risk are infants, children and elderly.

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4. Human Zinc Biochemistry

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Abstract. Zinc is nutritionally essential and indispensable to growth, development, and maintenance of human functions. There is hardly any cellular process that does not depend on zinc in some way. As a constituent of at least 2800 human proteins and with cellular concentrations of a few hundred micromolar, zinc has unparalleled significance in protein structure, enzymatic catalysis, and cellular regulation. The largest group of zinc metalloenzymes are proteinases. Zinc has a major role in the structural organization of protein domains that interact with DNA/RNA, other proteins, and lipids. Several dozen proteins control cellular and subcellular zinc homeostasis and re-distribution. The control is a prerequisite for regulatory functions of free zinc (II) ions. Fluctuations of cellular free zinc ion concentrations modulate the biological activity of a yet unknown number of additional proteins, suggesting roles of zinc in information transfer.

Keywords. Zinc biochemistry, catalytic, structural, and regulatory zinc sites, zinc signaling, zinc homeostasis, zinc finger, zinc enzymes, zinc metalloproteins

Introduction

A full account of zinc biochemistry has yet to be written because new structures and functions of zinc in proteins continue to be discovered. Major reviews on zinc biochemistry are almost twenty years old, and even a progress report is now ten years old [1-3]. I open this article by outlining four phases of discovery that advanced this nutritionally essential metal from obscurity to one of the most important elements of life.

First, Zinc biochemistry began in 1948 with the detection of zinc in carbonic anhydrase [4]. Ever since, direct chemical analysis established the presence of zinc in hundreds of enzymes and other proteins [5].

Second, in 1986, nine repetitive sequences were noticed in the transcription factor IIIA (TFIIIA) from the African clawed frog *Xenopus laevis*. They were shown to bind zinc via two cysteine and two histidine ligands and referred to as zinc fingers “because they contained zinc (Zn) and gripped or grasped the DNA” [6] (Figure 1). Zinc finger became a generic term for the structural role of zinc in many protein domains. Discovery research from now on often embraced *in silico* searches for possible binding sites in protein or nucleic acid sequences rather than either direct chemical analysis of zinc or measurements of whether the activity of a protein depends on zinc. With this bioinformatics tool, it was found that there are at least as many zinc proteins as there

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are zinc metalloenzymes, and it became possible to estimate the number of zinc proteins in a given genome. For humans, the estimate is about 2800 [7].

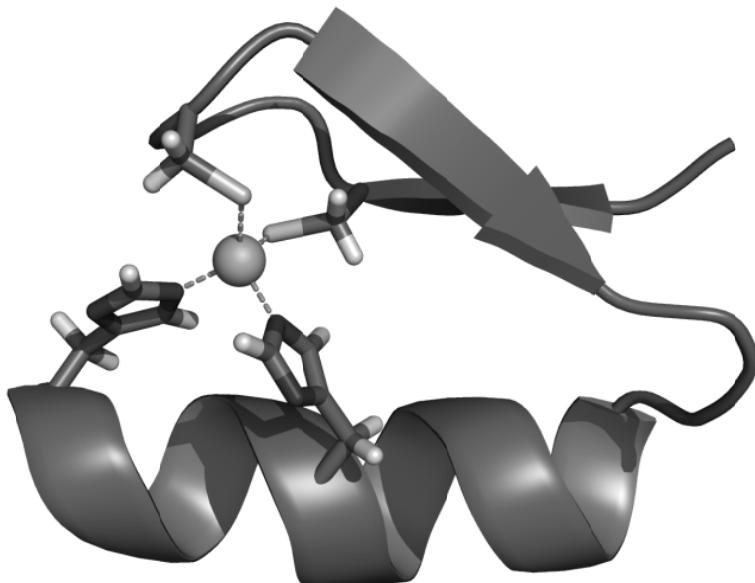


Figure 1. Zinc finger structure. The ligands of zinc are two histidines and two cysteines in a characteristic β - β - α protein fold (reproduced from Wikimedia Commons, GNU Free Documentation License)

Third, the large number of known and predicted zinc proteins generated interest in the question of how zinc is controlled and redistributed to fulfil all its cellular functions. The characterization of two families of zinc transporters and proteins involved in zinc-dependent gene transcription made it clear that cellular zinc is homeostatically controlled [8].

Fourth, the most recent development is the recognition that free zinc ions have functions independent of protein-bound zinc. Zinc ions are secreted from stimulated neurons into the synaptic cleft and affect the postsynaptic cell [9] (see chapter 18). Thus zinc ions are neuromodulators and *intercellular* signaling ions. Aside from neurons, many other cells have the capacity to secrete zinc ions [10]. *Intracellular* release of zinc ions amplified the concept of zinc as an information carrier by considering zinc ions as second messengers [11] (see chapter 6). The adage of “a galvanization of biology” describes succinctly the impressive and widespread catalytic, structural, and regulatory functions of zinc [12] (Figure 2).

While this plethora of zinc functions in proteins is already overwhelming, the question remains whether zinc has functions in other biomolecules. Amino acids, peptides, nucleotides, nucleic acids, and other biomolecules bind zinc ions. Since the physiological significance of these interactions remains unclear, this chapter will focus only on interactions of zinc with proteins as the present basis for human zinc biochemistry. It will address aspects of the zinc biochemistry of bacteria, plants, and single-cell eukarya only if necessary.

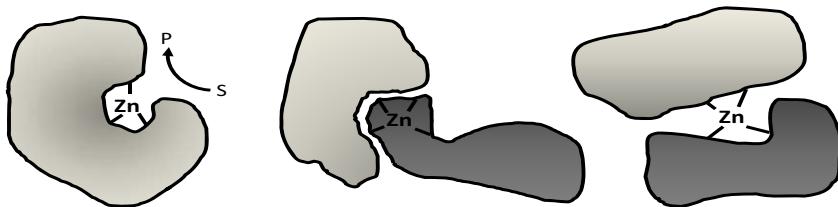


Figure 2. Functions of zinc in proteins. Zinc participates in catalysis in zinc enzymes (left), in protein-protein interactions where zinc-binding domains recognize other proteins (middle) or zinc bridges subunits of the same protein or different proteins (right). In addition to the structural roles that zinc has in these different binding modes, transient binding of zinc is involved in the regulation of the structure and function of proteins.

1. Zinc Coordination Chemistry in Proteins

A chemical basis for the functions of zinc in proteins is that zinc ions have no redox chemistry in human biology and remain in the divalent state, Zn(II); zinc ions are stereochemically flexible in their coordination; they are a Lewis acid, interact with both hard and soft donor atoms, and form rather strong complexes as indicated by the position of zinc next to copper in the Irving-Williams series [13]. With few exceptions, the donor atoms are the sulfur from cysteine (Cys), the nitrogens from the imidazole ring of histidine (His), and the oxygens from the side chains of glutamate (Glu) or aspartate (Asp). The combination of these few ligands in multiple binding modes, the use of several zinc ions that share ligands, or the use of zinc together with other metal ions generate a remarkable variety of coordination environments.

Critically important for the architecture of zinc sites and their properties is whether the donor atoms interact with amino acids in the secondary coordination sphere or involve even more global aspects of protein structure. For example, carbonic anhydrase binds zinc with picomolar affinity using only three protein ligands, whereas the *E. coli* zinc transporter Yiip binds zinc in a transport site much more weakly using four ligands. The reason for this difference in affinities is the extensive hydrogen bonding of the zinc ligands in carbonic anhydrase and the lack of hydrogen bonding in the zinc transporter [14,15] (Figures 3 and 4).

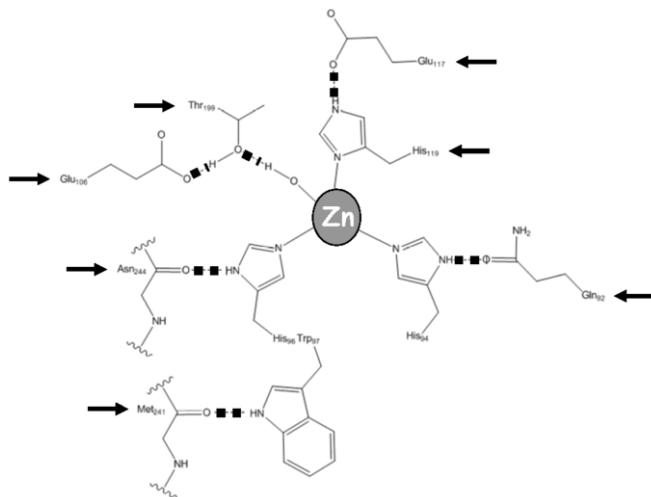


Figure 3. Zinc binding in human carbonic anhydrase II. Three histidines bind the catalytic zinc ion in carbonic anhydrase. Extensive hydrogen bonding in the secondary coordination sphere, including interactions with the zinc-bound hydroxide, stabilizes the interactions.

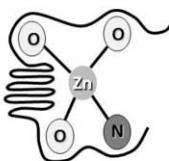


Figure 4. Zinc binding in the *E. coli* zinc transporter YiuP. In one of three zinc-binding sites, the transport site between transmembrane helices 2, 3 and 5 (site A), there are no interactions of the histidine and the three aspartate ligands beyond the primary coordination sphere. For details, see [15].

Zinc ions with the same set of donor atoms can serve widely different functions in proteins because the protein structure beyond the primary coordination sphere is also

important for function [16]. Thus inspection of the primary coordination sphere of a zinc site in a protein is a poor predictor of its function. Functional annotation is further complicated by the fact that enzymatic activity of some sites with features of catalytic zinc sites has not been found (sonic hedgehog [17]) and that some sites that look like structural sites are converted to enzymatic sites (matrix metalloproteinases [18]) or are active themselves (*E. coli* Ada protein [19]). In this article, I will follow the tradition of past reviews and present zinc sites in proteins as simple cartoons [20]. This oversimplification neglects the chemical bonds in the secondary coordination sphere and shows the zinc ions only in one state although many sites have dynamic coordination environments that would require a presentation of different states [16].

2. The Zinc Proteome and Metallome

Three-dimensional structures of zinc proteins supply templates of motifs with both characteristic donors coordinating zinc and numbers of amino acids between the ligands providing the donors, so-called spacers [20]. These motifs with their ligand signatures can be used in homology searches to identify putative zinc proteins in sequence databases. The power of this approach was illustrated for leukotriene A₄ (LTA₄) hydrolase (Figure 5).

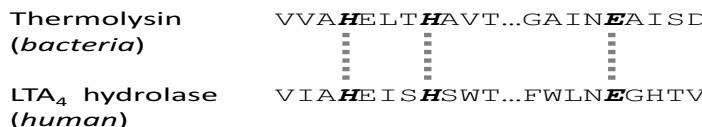


Figure 5. Ligand signatures in the primary structures of proteins. The two histidine and the one glutamate ligands of zinc and their typical spacing in the primary structure of bacterial thermolysin served as a template for a homology search in the primary structure of leukotriene A₄ hydrolase. The ligand signatures were found in the LTA₄ hydrolase, thus predicting that it is a zinc metalloenzyme and that it has peptidase activity.

Based on sequence homology to bacterial thermolysin, both the zinc-binding site and the peptidase activity of LTA₄ hydrolase were predicted and then confirmed [21,22]. In this way, many putative zinc proteins were identified. When genome databases became available and could be screened, estimates of entire zinc proteomes were obtained. For humans, the zinc proteome is estimated to contain about 2800 zinc proteins [7]. Since the list of motifs is incomplete, this approach provides a lower estimate. Additional zinc-protein interactions suggest that the actual number of zinc proteins is even larger [23]. Motifs are not necessarily specific for a particular metal. Depending on metal availability and control, the sites may contain a metal ion other than zinc [24]. Information about the affinity of a protein for zinc cannot be gleaned from ligand signatures. At present it is not straightforward to distinguish zinc

metalloproteins that bind the metal as a permanent cofactor and zinc-regulated proteins that bind zinc only transiently. The reasons are that proteins that interact with zinc transiently bind zinc with relatively high affinity and that many proteins are isolated or purified in the presence of chelating agents to preserve their biological activity and thus masked an inhibitory function of zinc. The ligand signatures of transient zinc-binding sites in proteins are largely unknown. Moreover, ligands can be located on different peptide chains, precluding their prediction from ligand signatures [25]. A definitive answer regarding the size of the human zinc proteome will require quantitative approaches that attempt to match the amount of zinc with the number of zinc proteins. At present, this goal is beyond reach of available methods for both detecting and measuring the large number of low abundance zinc proteins.

3. Zinc Enzymes: Catalytic Functions of Zinc

The main principle underlying the catalytic role of zinc in enzymes is the Lewis acidity of zinc in activating a substrate, a bound water molecule, or a protein ligand [26]. Very common are mechanisms where the activation produces a nucleophile, either a zinc-bound hydroxide or a zinc-bound thiolate (Figure 6).

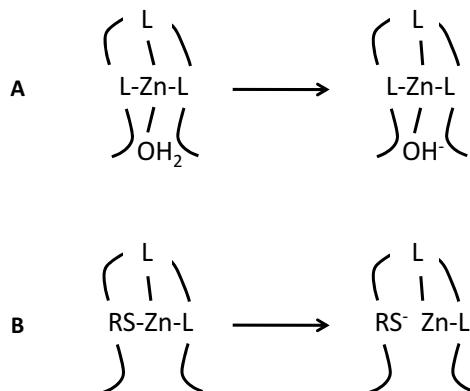


Figure 6. Catalytic functions of zinc in enzymes. The catalytic zinc activates either (A) a bound water molecule or (B) a thiolate ligand (RS^-) as nucleophiles.

The choice of ligands, ranging from three negative thiolates to three neutral histidinyl imidazoles, affects the formal charge at the metal ion, allowing considerable modulation of the Lewis acidity of the zinc ion. There are homoleptic complexes and complexes with any combination of oxygen, sulphur, and nitrogen donors (Figure 7).

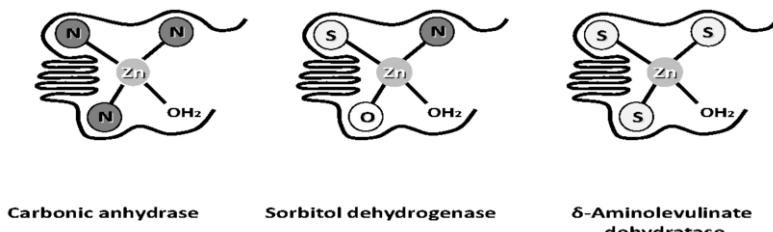


Figure 7. Three examples of coordination environments in zinc enzymes. Donors: N: nitrogen, S: sulfur, O: oxygen.

Enzymatic activity is often modulated by a second zinc ion, which has been named co-catalytic. The two zinc ions are usually bridged by an aspartyl side chain, but in alkaline phosphatase there is no ligand bridge. The typical coordination of zinc in metalloenzymes involves three protein ligands. Four ligands are also employed frequently.

Zinc enzymes are found in all six classes (oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases). Some generalizations about their functional significance can be attempted (Table 1). By far the largest group of zinc enzymes are metalloproteinases. It includes those needed for protein degradation and for processing proteins and generating biologically active proteins and peptide hormones. Zinc enzymes also feature prominently in phosphorylation signaling. They are conspicuously absent in the major pathways of energy and intermediary metabolism. However, they are present in the methionine cycle (betaine-homocysteine methyltransferase), and in committing enzymes of some pathways: in the eicosanoid pathway (LTA₄ hydrolase) and in the synthesis of tetrahydrobiopterin (GTP cyclohydrolase I), and in the Shemin pathway for heme synthesis (δ -aminolevulinate dehydratase).

Table 1. Examples of zinc enzymes and their ligand signatures

<u>1-zinc sites</u>	
<u>Proteinases:</u>	
Carboxypeptidases	Hx ₂ Ex ₁₀₃₋₁₂₄ H
Gluzincins with a characteristic HEx ₂ H motif followed by a third C-terminal Glu (E) ligand	Hx ₃ Hx ₁₈₋₅₈ E
Zincins (metzincins) with a characteristic HEx ₂ Hx ₂ Gx ₂ H motif followed by a C-terminal conserved methionine	Hx ₃ Hx ₅ H
<u>Other zinc enzymes:</u>	
Adenosine deaminase	HxHx ₁₉₉ Hx ₈₀ D
Alcohol dehydrogenases	Cx ₂₀ Hx ₁₀₆ C
Sorbitol dehydrogenase	Cx ₂₄ HE
Betaine-homocysteine methyltransferase	Cx ₈₁ CC
Carbonic anhydrases	HxHx ₂₂ H

Cytidine deaminase	Cx ₃₃ Cx ₂ C
Histone deacetylases	DxHx ₈₂₋₉₀ D
Glyoxalase I	Qx ₆₅ Ex ₂₆ Hx ₄₅ E
Phosphodiesterases	Hx ₃₅₋₃₉ HDx ₁₀₅₋₁₃₃ D
Protein phosphatases	Dx ₂₉₋₃₂ Nx ₄₇₋₅₉ Hx ₇₄₋₈₉ H
6-Pyruvoyltetrahydropterin synthase	Hx ₂₄ HxH
GTP cyclohydrolase I	Cx ₃ Hx ₆ C
Farnesyl and geranylgeranyl transferase	DxCx ₄₉₋₆₂ H

2-zinc sites

Dehydroprymidinase	HxHx ₂₅₆ D	Hx ₅₅ H
Leucine aminopeptidase	Dx ₇₆ DxE	Kx ₄ Dx ₁₇ Dx ₆₀ E
Methionine aminopeptidase	Dx ₁₀ Dx ₁₉₆ E	Dx ₆₈ Hx ₃₂ Ex ₉₄ E
Alkaline phosphatases	Dx ₃ Hx ₁₄ H	Dx ₄₉ Sx ₂₆₄ DH
Folate hydrolase (prostate-specific membrane antigen)	Hx ₉ Dx ₆₅ D	Dx ₃₇ Ex ₁₂₇ H

4. Zinc in Protein Structure

Zinc is of major importance in protein architecture. It serves structural functions to a greater extent than any other metal ion. As a redox-inert metal ion it bridges cysteine thiols and thus functions similar to disulfide bridges that are not favoured in intracellular proteins because of the reducing environment of the cytosol. An estimated 15,000 structural zinc sites exist in about 1000 human proteins, underscoring the fact that many domains contain more than one zinc ion, typically two, and that they are modular in terms of using several copies of zinc domains in one protein [27]. Using zinc in protein structure increases drastically the landscape of possible protein conformations and thus the potential for molecular interactions. Therefore, the availability of zinc may have been a major factor in the evolution of eukarya, which employ structural zinc sites much more extensively than prokarya [28].

The distinction between catalytic and structural zinc is somewhat arbitrary because catalytic zinc ions can also affect protein structure, and zinc in what may appear as a purely structural site can be chemical reactive and affect protein structure through reversible binding. Functionally, structural zinc sites are more variable than generally acknowledged. They are important for interactions between biomolecules, bridging of protein subunits, and control of protein structure [29]. The principles governing the coordination chemistry of structural zinc sites appear to be much simpler than for catalytic zinc sites. Virtually all structural zinc sites have four ligands in a tetrahedral geometry without a coordination site for a bound water molecule or another non-protein ligand. Zinc organizes protein structure within a protein, e.g. matrix metalloproteinases, in this case with oxygen and nitrogen donors (ligand signature: HxDx₁₂Hx₁₂H). By far the largest structural function of zinc, however, is in the organization of domains in zinc finger proteins (ZFPs). ZFPs recognize other proteins, DNA/RNA, or lipids. ZFPs have several modules, with up to 36 of them observed in one protein. The classical zinc finger domain has a β - β - α secondary structure with an N-terminal β -hairpin and a C-terminal α -helical region, each providing a pair of ligands. The ZF domains make contact to nucleobases in the major groove of DNA and wrap around DNA in a right-helical path. Other proteins with zinc-domains, sometimes also referred to as ZFPs, are involved exclusively in moderate to weak protein-protein interactions [27]. In ZFPs, the four ligands are preferentially cysteine (C) side chains with or without one or two histidines (H), i.e. CCCC, CCCH, and CCHH, with

virtually any permutation in the order in which the cysteines and histidines occur in the sequence (Figure 8).

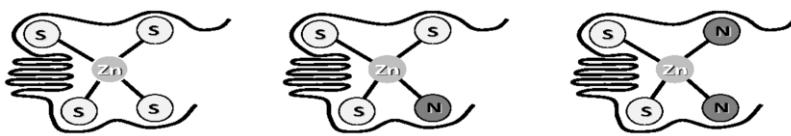


Figure 8. The three principal coordination environments in zinc finger proteins. Donors: N: nitrogen, S: sulfur,

The ligands can be close together in the sequence or spread over a wider range. Usually they form one or two pairs with the signature CxxC, sometimes with a longer spacer between the cysteines in the second pair. In addition to 1-zinc sites, there are domains with two, three, and even four zinc ions (Table 2). In 2-zinc sites, the ligands are often not used in the order in which they occur in the linear sequence, leading to interleaved binding patterns. In nuclear hormone receptors, such as the estrogen receptor, two zinc ions reside in one domain. One zinc ion is located in a region that is involved in dimerization of the protein while the other is in a region that is involved in its interaction with DNA. Another feature of some multi-zinc sites is the use of ligand bridges and thus fewer ligands than would be necessary for binding the zinc ions individually. Aspartate bridges with the two oxygen donors of the carboxy group; histidine bridges with the two nitrogen donors of the imidazole ring; and cysteine bridges with its sulfur donor. The nomenclature for ZFPs is confusing because it is not based on primary structure but on protein folds and it uses acronyms that refer to proteins in which the zinc sites were observed first. The sobriquets used, e.g. knuckle, box, ribbon, treble clef, RING = really interesting new gene, amuse rather than inform about function.

Table 2. Examples of zinc finger proteins and their ligand signatures

Domains:

1-zinc domains

Classical zinc finger motif, $\text{Cx}_2\text{Cx}_{12}\text{Hx}_{3-5}\text{H}$

such as in Sp1 and Krueppel transcription factors

GATA zinc finger, $\text{Cx}_4\text{Cx}_{17}\text{Cx}_2\text{C}$

Tumor suppressor p53 and its homologues p63 and p73, $\text{Cx}_2\text{Hx}_{58}\text{Cx}_3\text{C}$

2-zinc domains

PHD (plant homeodomain), ligand use not linear, cognate ligand: chromatin

$\text{Cx}_2\text{Cx}_{9-21}\text{Cx}_{2-4}\text{Cx}_{4-5}\text{Hx}_2\text{Cx}_{12-46}\text{Cx}_2\text{C}$

LIM (Lin-11, Isl1 and Mec-3), linear, cognate ligand: proteins

$\text{Cx}_2\text{Cx}_{17-19}\text{Hx}_2\text{Cx}_2\text{Cx}_2\text{Cx}_{16-20}\text{Cx}_2\text{C/H}$

A similar linear arrangement of ligands occurs in the MYND (Myeloid translocation protein 8, Nervy, and DEAF-1) domain, but its overall fold is different.

RING (really interesting new gene), not linear, role in ubiquitination
 $C_2C_{9-39}C_{1-3}H_{x-3}C_{2x}C_{4-48}C_{2x}C$

FYVE (Fab 1, YOTB, Vac 1, and EEA1), not linear, cognate ligand: phosphatidylinositol 3-phosphate
 $C_xC_x_1C_xC_x_4C_x_2C_x_6C_x_2C$

Protein kinase C (C₂-domain), not linear, cognate ligand: diacylglycerol
 $\text{Hx}_{12}\text{Cx}_2\text{Cx}_{13}\text{Cx}_2\text{Cx}_4\text{Hx}_2\text{Cx}_7\text{C}$

Nuclear hormone receptors, such as progesterone, androgen, estrogen, glucocorticoid, retinoic acid, thyroid hormone, vitamin D, and peroxisome proliferator-activated receptors

3-zinc domains

TAZ domain in the CREB-binding transcriptional adaptor protein CBP (p300)
 $\text{Hx}_3\text{Cx}_1\text{Cx}_4\text{C}$ $\text{Cx}_4\text{Cx}_8\text{Hx}_3\text{C}$ $\text{Cx}_4\text{Cx}_8\text{Hx}_3\text{C}$

4-zinc domains

C-terminal domain in metallothioneins (Figure 9)

Another principle in zinc-dependent protein structure is the bridging of peptide chains of the same or different proteins in sites that have been called protein interface zinc sites [30]. In this way, zinc organizes the quaternary structure, e.g. insulin hexamer, nitric oxide synthase dimer, or the quinary structure of proteins, e.g. the complex between the T-cell receptor CD4 and the protein tyrosine kinase Lck [31]. Zinc also organizes the structure of large aggregates as has been shown in SAM (sterile α -motif) domains of the Shank family of proteins, which serve as scaffolds for the postsynaptic density in neuronal junctions [32]. In these interactions, the same types of ligand donors as in catalytic and intermolecular structural zinc sites are used. Zinc sites with catalytic, structural, and regulatory functions have also been observed in membrane proteins.

5. Regulatory Zinc Sites in Proteins

5.1. Regulation of Cellular Zinc Homeostasis

The large number of zinc proteins made it clear that zinc concentrations need to be monitored and controlled homeostatically. At least four classes of proteins are involved in cellular zinc homeostasis: zinc importers and exporters, zinc sensor(s), and metallothioneins. The functions of these proteins are based on yet additional structural principles. The proteins employ coordination dynamics in their zinc-binding sites to control transient zinc binding.

ZnTs (SLC30A) transporters are a family of ten proteins, located on different cellular membranes. Four zinc ions are bound to the monomer of the *E. coli* Yiip homodimer, a protein with significant sequence homology to human ZnTs [15]. A zinc-binding site between the transmembrane helices appears to be the primary transport site. It is a tetrahedral site with donors from one histidine and three aspartates. Based on sequence homology, the donor atoms stem from two histidines and two aspartates in human ZnT1. Characteristic for this site is the absence of interactions of the donors with amino acids beyond the first coordination sphere (Figure 4). The ligands presumably dissociate to induce movement of the zinc ion. Less clear is the function of the other zinc ions in the hinge region and in the cytoplasmic domain. One is a dinuclear site with a bridging aspartate as found in enzymes, but in Yiip each zinc ion is bound by two histidine ligands that are constrained by outer shell chemical bonds

that couple zinc binding to interactions of the cytoplasmic domains. Zinc binding to the cytoplasmic domain leads to a conformational change that affects the orientation of the transmembrane helices. The third site has two histidines, one aspartate and a water molecule as ligands. Its function is unknown. Yip is thought to function as a Zn^{2+}/H^+ antiporter and as an allosteric protein with zinc-regulated zinc transport.

Zips (SLC39A). No 3D structure is available for any member of this family of 14 transporters. They are also located on different cellular membranes. Based on a comparison with the bacterial protein ZIPB, they are thought to be channels controlled by a membrane potential [33]. The zinc transporter will be described in detail in chapter 8.

Metallothioneins (MTs). Human MTs are a family of at least ten proteins that contain 60+ amino acids and bind up to seven zinc ions exclusively to the sulfur donors of cysteines in two zinc/thiolate clusters [34] (Figure 9).

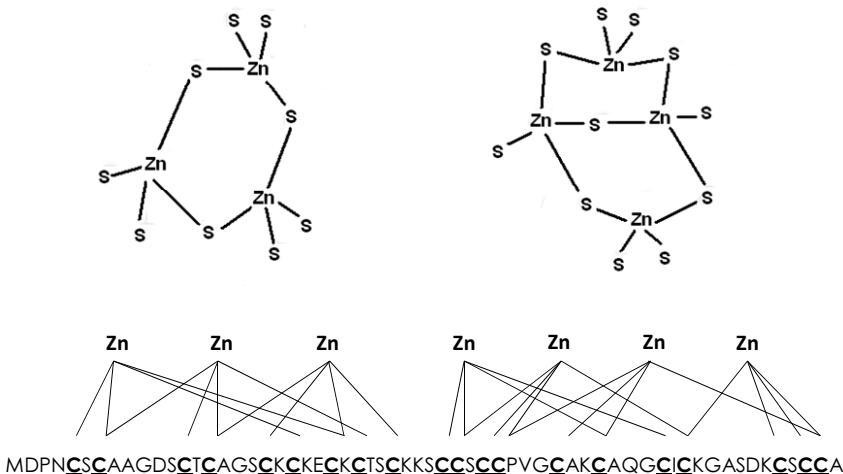


Figure 9. Metallothionein (MT) primary structure and zinc binding. The twenty cysteines in metallothioneins bind up to seven zinc ions. Each zinc ion is tetra-coordinate. Three cysteines bridge the zinc ions in the N-terminal 3-zinc/thiolate cluster (left). Five cysteines bridge the zinc ions in the C-terminal 4-zinc/thiolate cluster (right). The ligands are not used sequentially in the primary structure.

The functions of MT have been a matter of conjecture ever since the protein was discovered over 50 years ago [35]. MT did not fit into any of the classes of zinc proteins. It turns out that MT functions in a way that is indeed different from any other zinc protein. Recent insights into how cellular zinc is controlled (section 6.2) provide a context for the functions of MT in cellular zinc metabolism. Despite the binding of all seven zinc ions in tetrathiolate coordination environments, the affinities of MT-2 for zinc ions range from nanomolar to picomolar [36]. Commensurate with these affinities, MT can serve as both a zinc acceptor and a zinc donor and control the availability of cellular zinc over a range of concentrations [37]. In the cell, MTs occur in different

states that depend on zinc availability and redox poise. In contrast to other zinc proteins, the sites of which are generally fully occupied with zinc ions, the zinc-binding sites of MT are not fully occupied under all conditions and neither are all of its cysteines reduced, as the structural model with seven zinc ions implies. In MT, thiolate ligand-centered chemistry modulates the zinc-binding capacity [38]. In this way, MTs are redox proteins and can transduce redox signals into zinc signals: signal → reactive species → MT → $[Zn^{2+}]_i$ signal → target [39]. The zinc-donor potential of MT increases under more oxidizing conditions while its zinc-acceptor potential increases under more reducing conditions and by de novo synthesis of the apoprotein thionein (Figure 10).

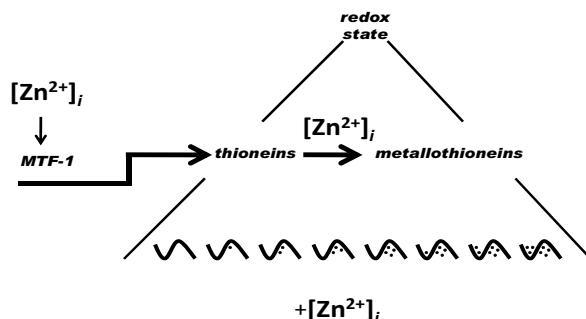


Figure 10. Function of metallothioneins in zinc metabolism. The expression of metallothionein genes – with the exception of MT-3 – is controlled by the Zn^{2+} sensor MTF-1 and multiple signal transduction pathways (not shown). The cellular redox state affects the availability of zinc ions and the zinc-binding capacity of thioneins. Depending on zinc availability and redox state, metallothioneins are loaded with up to 7 zinc ions. The metal-loaded form is metallothionein and the metal-free form is thionein.

Within cells, MT translocates to the mitochondrial intermembrane space and shuttles between the nucleus and the cytosol. It is also secreted from cells, and is taken up by cells through megalin-receptor-mediated endocytosis [40]. So far, no protein other than MT has been found to function in temporary cellular zinc storage. The micromolar concentrations of MT in most cells make sufficient zinc available in a deliverable form to meet the demand of many cellular functions for zinc.

MTF-1. Metal-response element binding transcription factor-1 (MTF-1) is an essential protein that controls zinc-dependent gene expression [41]. Its 3D structure is unknown. It is a sensor for elevated zinc ion concentrations and controls the expression of antioxidant, developmental, and zinc homeostatic genes [42]. It interacts with DNA via six zinc fingers, one or two of which are thought to participate in zinc sensing [43].

5.2. Zinc in Cellular Regulation

Whether zinc ions can serve regulatory functions depends on the concentration at which they are available, an issue that is linked to the equilibria between protein-bound and free zinc ions and how cellular zinc itself is regulated. Regulation requires

transient zinc binding and processes that either make zinc ions available or restrict their availability.

Three pathways increase free zinc ion concentrations. First, zinc ions are released from exocytic vesicles into the extracellular space as observed originally in synaptic vesicles of certain glutamatergic neurons [9]. One target is the postsynaptic N-methyl-D-aspartate (NMDA) receptor, the NR2A subunit of which is inhibited by low nanomolar concentrations of zinc ions [44]. Secretion of zinc ions from vesicles also occurs in a number of exocrine and endocrine glands, notably the pancreatic islets, where zinc is needed for insulin storage in β -cell granules and secreted together with insulin [10]. Second, zinc is released from a store in the endoplasmic reticulum (ER) via control of Zip7 [45,46]. And third, modification of sulfur donors in zinc/thiolate coordination environments of proteins leads to dissociation of zinc ions [38] (Figure 11). In contrast, ZnTs and MTs restrict the availability of zinc ions.

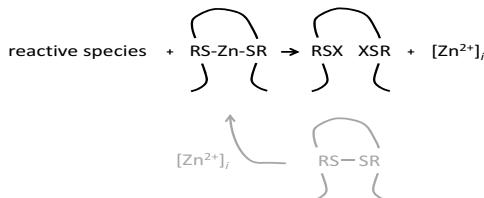


Figure 11. Reactivity of zinc/thiolate coordination environments in proteins. Reactive species modify the properties of the sulfur donors of zinc/thiolate (RS-Zn) coordination environments in proteins, resulting in derivatives of the thiolate (RSX) and dissociation of zinc(II) ions. If the product of the reaction is a disulfide (RS-SR, shown in grey), the disulfide can be reduced and the zinc-binding capacity of the protein restored.

The resting cellular free zinc ion concentrations in human cells and cell lines are estimated to be a few hundred picomolar [47,48]. However, the total cellular zinc concentration is a few hundred micromolar and thus at least six orders of magnitude higher. It is also about two orders of magnitude higher than the extracellular zinc concentration. The large ratio between bound and free zinc is a consequence of zinc proteins binding zinc with picomolar affinities. Globally, zinc ion concentrations can increase transiently above one nanomolar [49,50]. At the site of their release, local zinc ion concentrations may be even higher. The expression of such zinc ion signals and their use as biological effectors depend on the cytosolic zinc buffering capacity and on the activities of transporters that remove zinc ions to restore the steady state [51]. The combination of metal buffering and metal fluxes is called muffling [52]. Muffling reactions involve the binding of the surplus of zinc ions to MTs and moving zinc ions to an organelle or out of the cell [53]. The compartmentalization and vesicular traffic of zinc ions is a central aspect of the cellular homeostatic control of zinc and is more akin to calcium than to any of the other transition metals.

With zinc ion fluctuations reaching concentrations of only a few nanomolar on average, one would expect that the targets of zinc ions have affinities commensurate with these concentrations. It is unknown how many targets there are and whether the sites targeted have structural features in common. At least four types of targets are thought to be modulated by fluctuating zinc ion concentrations. First, the occupancy of co-catalytic zinc sites modulates the activity of zinc enzymes. Second, some enzymes

that are not recognized as zinc enzymes are zinc-inhibited with affinities in the low nanomolar range or even lower, e.g. erythrocyte Ca^{2+} -ATPase with a K_i value of 80 pM [54,55]. Resting zinc ion concentrations of 24 pM in erythrocytes suggest tonic inhibition of this enzyme [56]. Release of zinc from an ER store could provide high enough local zinc ion concentrations to inhibit protein tyrosine phosphatase-1B (PTP-1B), which is tethered to the ER membrane and has a K_i value of 16 nM for zinc [57]. Third, metal-response element (MRE) binding transcription factor-1 (MTF-1) is an established target that senses a few nanomolar zinc ions [43]. Fourth, given the low availability of cytosolic zinc ions and their control, it is expected that zinc controls protein-protein interaction at protein interface zinc sites, and perhaps the organization of large protein assemblies.

Much weaker interactions of proteins with zinc may be physiologically significant if the proteins are not cytosolic and stored and released together with zinc. Examples are carboxypeptidase A, a zinc proteinase, and kallikreins, which are serine proteinases. Both have inhibitory sites with low micromolar affinities for zinc [25]. Subcellular compartmentalization allows much higher local zinc ion concentrations than in the cytosol. Once the zinc-inhibited enzymes are released from the cell, dilution into a medium of much lower zinc ion concentrations will activate them.

From this overview, it appears that regulation of zinc is a prerequisite for regulatory functions of zinc ions. An article written about 30 years ago answered the question “Zinc: what is its role in biology?” by suggesting a fundamental role of zinc in inhibitory control, and by pointing out the vesicular sequestration of zinc and the complementarity of zinc and calcium biology in cellular regulation [58]. The recent findings are consistent with these conclusions that were based on an extraordinary vision.

6. The Metabolic and Molecular Zinc/Redox Links

In addition to the function of zinc in enzymes, protein structure, and protein regulation, zinc has a general function in redox metabolism [59,60]. It is paradoxical to consider zinc an antioxidant because zinc(II) ions are redox-inert in biology. Since zinc ions are involved indirectly in redox biochemistry, it is more appropriate to refer to zinc as a pro-antioxidant. However, this pro-antioxidant potential is expressed only in a narrow range of zinc concentrations. Outside this range pro-oxidant conditions prevail because oxidative stress is observed if zinc ion concentrations are too low or too high (Figure 12).

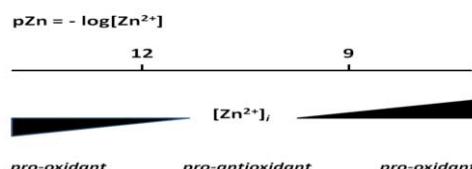


Figure 12. Indirect redox functions of biologically redox-inert zinc(II) ions. In a physiological range of nanomolar to picomolar zinc ion concentrations ($p\text{Zn} = - \log[\text{Zn}^{2+}] = 9-12$), zinc ions are pro-antioxidants. Concentrations of zinc ions outside this range have a pro-oxidant effect.

These dual functions of zinc are a prime example of the limited validity of qualitative considerations in biology when quantitative considerations reveal opposite effects. Molecular and metabolic links account for the mechanisms of these indirect redox functions of zinc ions. MT is a central metabolic link between zinc and redox metabolism [61]. The redox-active sulfur donor atoms in the zinc/thiolate coordination environments determine zinc dissociation and association. In this way, zinc availability is under control of redox metabolism. But the relationship between zinc and redox is reciprocal because zinc availability also affects redox metabolism. Under physiological conditions, zinc is a pro-antioxidant. Its binding to cysteine sulfhydryls protects the cell against injury induced by reactive substances. Also, zinc is involved in the expression of antioxidant and other proteins that are under the control of MTF-1 and Nrf-2 transcription factors [62,63] (Figure 13).

Zinc deficiency and zinc overload are pro-oxidant conditions. Zinc deficiency provides insufficient protection of sulfhydryls and compromises the expressions of genes encoding antioxidant enzymes. High zinc ion concentrations generate oxidative stress by inhibiting mitochondrial respiration and antioxidant enzymes, such as thioredoxin and glutathione reductases.



Figure 13. Protective functions of zinc ions. Zinc ions affect the expression of genes that depend on the Nrf-2 or Mtf-1 transcription factors. In this way, zinc ions elicit a protective response in the cell against various types of insults such as stress from redox-active agents, metal ions, and xenobiotics. Any surplus of zinc ions is bound to proteins or sequestered in organelles.

7. Conclusion and Perspectives

As one of the major elements of life the significance of zinc for health and disease is emerging. With cellular concentrations as high as a few hundred micromolar, zinc can hardly be considered a trace element. Since the last major accounts of zinc biochemistry appeared [1-3], significant discoveries continue to enhance the importance of zinc biochemistry. New functions of zinc-protein interactions in cellular regulation add to the already impressive range of functions in at least 2800 human zinc proteins. Yet, as discussed in these earlier reviews, gauging the full impact of zinc on cellular physiology is an ongoing process. At present, the large databases on structures and sequences of zinc proteins do not translate into an integrated view of zinc functions. At least 30% of the human zinc proteins remain to be functionally annotated, leaving ample room for future discoveries. With a focus on molecular and cellular rather than elemental biology, the fundamental importance of zinc is marginalized in most general textbooks of biochemistry, if treated at all. Of even greater concern is that perturbations of zinc metabolism are rarely considered in general medical practice despite of the widespread occurrence of nutritional zinc deficiencies in certain populations and inborn errors of zinc metabolism that predispose individuals to nutritional, toxic, or pharmacological exposures that compromise the homeostatic control of zinc [64,65].

A major unresolved issue is how all the zinc proteins acquire their zinc at the right time and whether there is any hierarchy in the organellar, cellular, and subcellular distribution of zinc. It is not known whether the cell prioritizes certain processes under zinc-limited conditions. Are some functions sacrificed for the preservation of other more vital ones? When zinc is lowered or increased, are some proteins affected preferentially or are all proteins affected simultaneously? Until now, the sheer number of zinc-dependent proteins mostly precluded an association of zinc deficiency with one or several specific biochemical processes. But our inability to make such associations should not distract from acknowledging the fundamental and critical roles that zinc has in maintaining human functions.

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5. Zinc in Mammalian Cell Cycle and Cell Death

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Abstract. Life has evolved around Zn and so apparently has the regulation of cell division and cell death. An increase in cytosolic Zn during mid-G1 is essential for the induction of cyclins and other gene products required for the cell to prepare for replication of its DNA. A second requirement for Zn precedes mitotic division of the cell. A role for the action of specific Zn transporters in mitosis is rapidly emerging. The relationship of Zn to cell death is intriguing as this metal ion can both suppress and promote apoptosis, depending upon factors which are still poorly understood. Zn protects many types of cell from oxidant-induced apoptotic cell death by, amongst other things, inhibiting events leading to activation of the caspase executioner proteins. Recent studies showing a role for Zn in macrophage phagocytosis suggests impaired efferocytosis as an alternative mechanism by which apoptotic cells can accumulate in the tissues in Zn deficiency. Under some circumstances, Zn acts as a potent inducer of apoptosis, such as in neurons dying during seizures and brain injury where it contributes to pathogenesis. Other emerging mechanisms of cell death, including autophagy and pyroptosis, are also influenced by Zn and Zn deficiency. In addition to the above roles of Zn as either a suppressor or promoter of cell death, a general feature of apoptosis and autophagic cell death is a substantial increase in fluorophore-detectable, labile Zn within the dying cell's cytoplasm or vacuoles, respectively; the significance of this Zn release is not known. The dual roles of Zn in regulating cell proliferation and cell death point to a pivotal role for this metal ion in tissue homeostasis with important implications for diseases in which the delicate balance between cell birth and cell death goes amiss.

Keywords. Cell cycle, cell death, zinc, zinc transporter, tissue homeostasis

Introduction

Normal tissue homeostasis requires the coordinated regulation of two major cellular processes: - cell division and cell death. Aberrations in their regulation contribute to the pathogenesis of many diseases ranging from cancer at one end of the spectrum to degenerative disease at the other end. As zinc (Zn) is a regulator of multiple signalling pathways in both cell division and cell death, this metal ion has an important role to play in ensuring normal tissue homeostasis. Cells rapidly mobilize Zn following awakening from quiescence as a prelude to the requirement for this metal ion in nucleic

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acid synthesis, cyclin synthesis and other events [1-3]. By contrast, cells release Zn when they die by apoptosis and autophagy [4-6].

Prior to a detailed discussion of the separate effects of Zn ions on the opposing processes of cell division and cell death, it is important to bear in mind the following caveat concerning interpretation of some of the findings. There is cross-talk between signalling processes mediating cell growth and cell death. Stimulation of proliferation by growth factors and other agents often results also in suppression of cell death [7]. Induction of apoptotic cell death in proliferating cells often involves and requires also cell cycle arrest [8]. Furthermore, apoptotic cell death in tissues is often accompanied by compensatory tissue proliferation [9]. Therefore, particularly when one considers the effect of Zn on tissue homeostasis, it is not always clear whether the primary effect of Zn is on cell proliferation, with a consequent secondary or compensatory effect on cell death, or vice versa, or whether Zn is directly interfering with both cell proliferation and cell death mechanisms, simultaneously. When we say that in Zn deficiency, cell proliferation is decreased, we cannot be absolutely certain that the decrease in proliferation we measure is not simply the consequence of enhanced cell death. While one should keep this caveat in mind when interpreting effects of Zn ions on cell proliferation and cell death assays, this chapter will show that Zn ions do interact equally with cell proliferation signalling pathways and cell death signalling pathways.

Although Zn fixed tightly in metalloenzymes and Zn finger transcription factors has major housekeeping roles in cell growth and cell death, the dynamic regulation of these processes by this metal ion is more likely to fall under the control of the more exchangeable or labile pools of Zn. This chapter begins with a discussion of labile Zn, its definition, detection, manipulation and significance. The next part of the chapter deals with the involvement of Zn and its transporters in cell cycling and division. The third part discusses the role of Zn in cell death, especially the process of gene-regulated cell death (apoptosis), but also necrosis and more recently discovered mechanisms of cell death such as pyroptosis and autophagy.

1. Concept of Labile Zinc and its Relevance to Tissue Homeostasis

Classically, we think of organ and tissue Zn as existing in two forms: a fixed pool comprising most of the Zn very tightly bound within metalloenzymes and Zn finger transcription factors and a labile pool comprising a minor and variable pool of loosely bound or free Zn ions. This classification has functional relevance since we can think of the fixed pool as largely mediating house-keeping roles within cells such as enzyme catalysis and gene expression, while the labile pool is better placed to interact reversibly with signalling pathways and thereby control dynamic events such as secretion, apoptosis and neurotransmission [10]. It has methodological implications also since many of the histochemical stains and fluorescent techniques to detect tissue Zn selectively detect the labile pools; detection of fixed pools often requires cell homogenization or other means of disruption [11]. The classification is also relevant from a disease perspective since nutritional or secondary deficiencies of Zn are more likely to affect the more exchangeable labile pools.

This classical concept of fixed and labile pools of Zn becomes a bit fuzzier when we consider tissues undergoing rapid cell turnover. There are at least two major reasons for this. Firstly, in rapidly dividing tissue, although Zn may still be tightly bound within

some metalloproteins, the proteins themselves will turnover as rapidly as the cells divide. In this situation, Zn deficiency will have significant impact on the functions of these Zn enzymes and transcription factors. Secondly, redox events are more common in tissues with rapid turnover. Changes in the redox state of the cell occur in dividing cells (where there is a shift towards a more reduced cytosol) and in dying cells (where there is a shift towards a more oxidized cytosol) [12]. Redox potential affects Zn homeostasis because the preferred binding of Zn ions to protein sulphur (in cysteine) and nitrogen (in histidine) is sensitive to oxidation (see chapter 4). Therefore, one can detect quite dramatic changes in intracellular labile Zn levels when cells are treated with an oxidant such as nitric oxide [13] or when cells undergo apoptosis [4].

2. Zn and Cell Cycle

2.1. Overview of Mammalian Cell Cycle

The cell cycle is separated into a number of sequential phases, G1 (gap phase 1), S (DNA synthesis), G2 (gap phase 2) and M (mitosis). Entry into each phase is regulated by check-points and other signals, so that one phase is satisfactorily completed before the next begins [14]. Entry into G1 can occur either from G0 or, for rapidly dividing cells, immediately following M phase of the previous cell cycle. Where the tissue is undergoing slow turnover, completion of M phase is followed by exit from cycle into G0. Passage through the cell cycle is controlled by the actions at specific stages of heterodimeric complexes of two types of proteins, cyclins and cyclin-dependent kinases [15]. Induction and degradation of specific cyclins at various stages of the cycle allows tight regulation of the kinase activities. It should be noted, however, that recent gene knockout studies have thrown doubt on some of the dogma, especially relating to the role of cyclin D-dependent kinases in G1 [16]. Our understanding of cell cycle regulation therefore remains incomplete at this point of time. The following provides a simplified overview of generally-held concepts of the roles of different cyclin-kinase activities in the mammalian cell cycle, as a background to understanding better how Zn impacts on some of these pathways.

2.1.1. G1 Phase

During the G1 phase, a cell becomes committed through signalling pathways and new gene expression to enter S phase. Once commitment has been completed and the cell passes a check point late in G1, it now is no longer dependent on extracellular growth signals or other factors [14]. Passage through this checkpoint is controlled by the interplay between a number of cellular proteins, that may be either stimulatory or inhibitory with regards to subsequent entry into S phase. New cell division requires the action of a family of transcription factors known as the elongation 2 transcription factors (E2Fs). These are responsible for expression of a number of critical target genes required for passage into and through S phase. In a non-dividing cell, E2Fs are held inactive by binding of an inhibitory protein pRb, originally discovered as a tumour-suppressor factor for malignant retinoblastoma [17]. pRb may also block passage through its binding to histone deacetylases and other chromatin-associated proteins [18].

Critical to progression through G1 into division is the action of a factor that was initially termed MPF (an acronym for mitosis-promoting factor). It is now known that MPF is not a single protein but a complex of two types of proteins, a cyclin (which may be D, E or A) and a cyclin dependent kinase (CDK, usually CDK4 or CDK6). Cyclins are induced in response to growth factor stimulation during G1 and bind to and activate pre-existing CDKs. These complexes in turn phosphorylate pRb, by sequential actions beginning with cyclin D-CDK4 and cyclin D-CDK6. This phosphorylation results in partial inactivation of pRb and sufficient release of E2F to mediate induction of cyclin E. Cyclin E then binds to and activates CDK2 and induces further phosphorylation of pRb which results in complete detachment from the E2Fs, allowing these transcription factors to fully activate genes required for DNA replication and mitosis [19].

Once formed, the cyclin-CDK complexes are themselves negatively regulated by binding of inhibitory proteins, principally the INK4 (inhibitors of CDK4) and the Cip/Kip family of inhibitors [20]. INK4 proteins bind and inhibit the catalytic activity of CDK4 and CDK6. The Cip/Kip family, which includes p21Cip1, p27Kip1 and p57Kip2, bind both to cyclin and CDK subunits and suppress the activities of the respective kinases. Of particular interest has been the role of p53 which induces p21Cip1 activity in response to DNA damage [21]. Therefore, a cell which has begun to prepare for entry into S phase, by turning on expression of the appropriate cyclins and forming catalytically-active cyclin-CDK complexes, may still be prevented from passage into S-phase by downstream negative signals that induce expression of the INK4 or Cip/Kip proteins. The cell will remain arrested at the G1/S transition. Cancers may utilize disruption of cell cycle inhibitory processes (e.g. by loss of pRb or inactivation of INK4, Cip/Kip and p53) to promote tumor growth.

2.1.2. S Phase

Cyclin E-CDK2 is important for phosphorylation of the pre-replication complex to initiate DNA replication. Once cells enter S phase, cyclin E is degraded by ubiquitin-dependent proteolysis [22]. Cyclin A now assumes a major role. It binds to CDK2 and activates its kinase site. One target of this kinase is E2F which now becomes inactive. Other targets for this kinase may be involved in DNA synthesis since cyclin A-CDK2 localizes to sites of DNA replication [23].

2.1.3. G2 and M Phase

G2 contains a checkpoint that responds to DNA damage and allows DNA repair before entry into mitosis. As with G1/S arrest, p53-dependent induction of p21 is one of the mechanisms to arrest cells at the G2/M checkpoint [24]. Once through this control, CDK1, in association with cyclins A and B, assumes a critical role in M phase [25]. Targets of CDK1 include lamins, histone H1, and the mitotic spindle. Degradation of cyclins A and B accompany exit from M phase.

In summary, the cell cycle consist of the highly dynamic events of DNA replication and mitotic cell division, separated by stages G1 and G2 which prepare the cells for S and M phases and which contain very important checkpoints G1/S and G2/M to ensure accurate genomic DNA replication.

2.2. Zinc and DNA Synthesis

2.2.1. Early Studies of Effects of Zn Deprivation In Vitro and In Vivo on Nucleic Acid Synthesis

The first insights into the importance of Zn for cell cycling came from a series of studies by the Lieberman group at the University of Pittsburgh in the early-mid 1960s [26-29]. While investigating the requirements for culture of primary rat kidney cortex cells, they found that the divalent metal ion chelator ethylenediamine tetra acetic acid (EDTA) completely suppressed DNA synthesis and this could be reversed by Zn ions. None of the other metal cations tested (calcium, magnesium, cadmium, cobalt, iron, manganese or nickel) had any effect. Interestingly, there were no effects of EDTA on rates of synthesis of RNA and protein. However, both DNA formation and the late G1 increase in specific activities of DNA polymerase and thymidine kinase were suppressed. This indicated a critical requirement for Zn ions in the activity of DNA synthesizing enzymes and subsequent DNA synthesis in S phase.

A similar requirement for Zn ions was next shown in a rat liver model, in which removal of up to two thirds of the liver during surgery resulted in a marked stimulation of DNA synthesis when the remaining liver cells were isolated and cultured for 22 hrs. Administration of EDTA by infusion *in vivo* suppressed this DNA synthesis and once more only Zn ions reversed the effect of EDTA. Magnesium ions boosted the effect of Zn but had no effect by themselves. None of the other metal cations tested had any effect. Again there was no effect of EDTA on RNA synthesis [26].

These studies also made two key observations which are yet to be fully understood. Firstly, EDTA only suppressed DNA synthesis when added before or during a window of several hrs corresponding to mid-G1. Once this window had passed, there was no longer a requirement for extracellular Zn ions. Secondly, when considering a range of cultured cells, the authors concluded that the sensitivity to Zn depletion was much higher in freshly obtained primary cells than in later passage cultures or in immortalized cell lines. One conclusion is that cultured cells adapt over time to the extracellular Zn concentrations. In hindsight, we may now wonder whether both observations reflect changes in expression and/or activity of cell cycle related Zn transporters.

The rat liver study described above suggested also that one of the sequelae of *in vivo* Zn deficiency in animals and man may be impaired tissue DNA synthesis and this could contribute to the growth retardation and tissue atrophy associated with severe Zn deficiency. It was pointed out that whereas the EDTA infusion model resulted in loss of 70% of liver Zn, dietary Zn deficiency rarely depleted more than 30% of liver Zn [30]. The effects of a low Zn diet on DNA synthesis in the liver of rats was tested by a number of groups. Conflicting results were obtained which were attributed to a number of complicating factors including age of the rats, effect of Zn deficiency on appetite and effects of Zn deficiency that arise secondarily [30]. In a carefully controlled rat experiment, Williams and Chester showed that a low Zn diet resulted in an early significant decline in DNA synthesis in liver, testes and other tissues [30].

The studies in the 1970s on the suppressive effects of Zn deprivation on lymphocyte proliferation [31; 32] not only confirmed the findings in other cell types but also suggested a mechanism that might at least contribute to the deficits in immune responsiveness associated with Zn deficiency. Interestingly, both of these studies reported a synergistic action between Zn and ferrous ions in restoring DNA synthesis in

the Zn-depleted lymphoid cultures. This has practical implications since Zn deficiency is often accompanied by iron deficiency [33]. It should be noted that while all studies have shown a primary effect of Zn on cell proliferation, the different models have shown different influences of other metal cations.

2.2.2. Presence of Zn in Nucleic Acid Synthesizing Enzymes

The finding of stoichiometric quantities of Zn in DNA and other nucleic acid synthesizing enzymes from a variety of species and the demonstration that Zn chelators interfered with DNA synthesis, led to the general belief that the requirement for Zn during cell division simply reflected a Zn requirement for nucleic acid synthesis [30; 34-38]. It was suggested that the abnormalities in the fetus arising from maternal Zn depletion in rats may result from impaired DNA synthesis in the developing fetus [36; 37] (see chapter 15). Of particular interest were the Zn-dependent enzymes mediating DNA synthesis [39-41].

In view of previous data that a number of metabolic enzymes such as carbonic anhydrase and alcohol dehydrogenase are true Zn metalloenzymes containing stoichiometric quantities of Zn required for catalysis or regulation [42; 43], it seemed plausible that DNA polymerases and other nucleic acid-synthesizing enzymes might also be Zn metalloenzymes. Based on previous work showing that terminal deoxynucleotidyltransferase, an enzyme with similar catalytic properties to DNA polymerase 1 was dependent on Zn ions, it was demonstrated that *E coli*, sea urchin and chick embryo DNA polymerases contained up to 4 Zn atoms per molecule, depending on species; in addition, catalytic activities of these enzymes were suppressed by Zn chelators [39; 44; 45]. It was proposed that Zn mediated the binding of the enzymes to DNA and that Zn chelators might be useful as adjuncts to chemotherapy, if they could be targeted to tumours. The presence of Zn in nucleic acid synthesizing enzymes is now known to be a more general phenomenon including also the DNA-dependent RNA polymerase from *E coli* [46].

While these studies showed a definite requirement for Zn for many nucleic acid-synthesizing enzymes, subsequent research described below identified major requirement(s) for Zn well prior to the time of nucleic acid synthesis.

2.3. Zn Requirement during Mid-G1

Time-course studies with lymphocytes confirmed earlier findings that cells required Zn in a window several hours after leaving quiescence but prior to DNA synthesis (Fig 1) [32]. This was explored in more detail in a subsequent study from the group looking at effects of *in vitro* Zn chelation with diethylene trinitriolo pentaacetate (DTPA) on DNA synthesis in synchronized cultures of 3T3 cells [47]. Two temporally distinct requirements for Zn ions were observed. The first was within the period from 8 h after stimulation of quiescent cells with serum until 3 h before the start of S phase. Based on studies in 3T3 cells with inhibitors of RNA and protein synthesis, it was proposed that Zn-dependent gene expression was required for successful progression of G1 cells into S phase [48]. The second requirement for Zn occurred during S phase (Fig 1) and was essential for passage of cells through the subsequent stages of G2 and M [47].

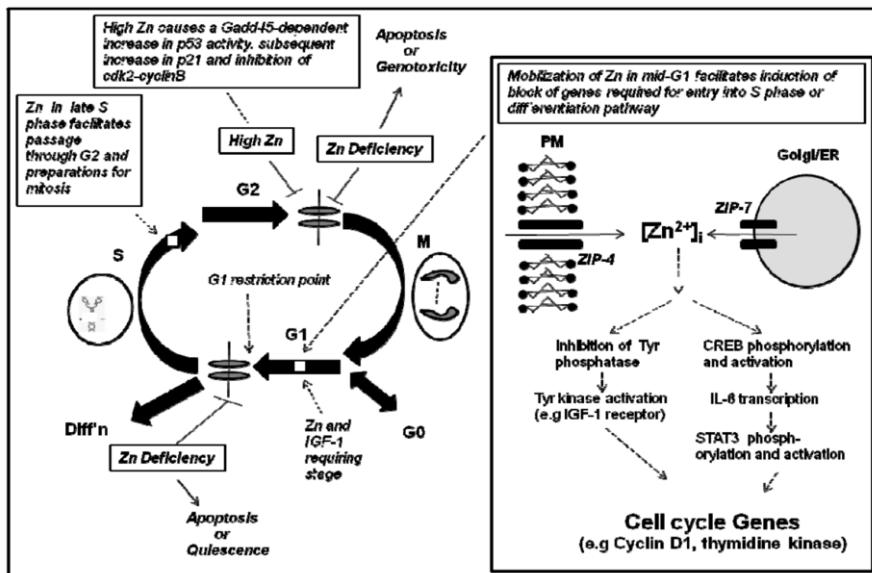


Figure 1. Roles of Zn in cell-cycling. Mobilization of Zn is essential during the IGF-1 sensitive stage of G1 and during late S phase. Deficiency of Zn during these periods results in arrest at G1/S and G2/M checkpoints, respectively, and may result in return to quiescence, apoptotic cell death or genotoxicity. High Zn also arrests cells at G2/M via p53 and p21-dependent inhibition of cdk2-Cyclin B. **Inset:** Zn mobilization during G1 permits the induction of genes for cyclin D1, thymidine kinase and other proteins required for entry into S phase. In G1, intracellular Zn increases by influx of Zn via ZIP-4 and by Zn release from endoplasmic reticulum or golgi via ZIP-7. The resulting Zn flux inhibits tyrosine phosphatases, leading to activation of IGF-1 receptors (and other tyrosine kinases), as well as by CREB-dependent increases in interleukin (IL)-6 and STAT3 activation. Whether these two pathways converge and whether the source of the Zn flux affects which of the signalling pathways predominates is not clear.

2.4. Zn Requirement during G2

Both insufficient Zn and excessive Zn arrest cells in G2 at the G2/M checkpoint. Mammalian cells arrested at G1/S by hydroxyurea and then released from arrest by adding fresh serum-containing medium, completed S phase, G2 and M normally. However, where release from arrest was conducted in the presence of DTPA, they completed S phase but arrested in G2. In these experiments, DTPA was added with sufficient iron so that the arrest in G2 was not due to iron deficiency. Re-addition of Zn allowed cells to pass through G2 normally. This experiment also confirmed that DNA synthesis in S-phase was not directly affected by inadequate Zn [2]. It supported earlier work done on *Euglena gracilis*, showing that when Euglena were allowed to grow in low Zn medium, the cells mainly arrested in the G2 phase [49].

More recently, studies of the growth-suppressive effects of high extracellular Zn in bronchial epithelial cultures have shown blockage at G2/M transition, mediated by Zn-dependent enhanced expression of p53, consequent up-regulation of p21 and then p21-mediated inhibition of Cdc2/Cyclin B [24]. Further studies from the group showed that the suppressive effects of Zn were accompanied by enhanced phosphorylation of p38 and p53 and mediated by the cellular stress sensing protein Gadd45 since silencing of Gadd45 in the epithelial cells prevented the G2/M arrest [50].

2.5. Zinc Requirement for Differentiation

Cells which arrest at G1/S may revert to quiescence or undergo cell death (discussed in a later section). Once through the G1/S check-point, a cell may undergo two fates (depending on the cell type and other factors): entry into S phase and cell cycle or entry into a differentiation pathway. Chesters and colleagues introduced the interesting concept that, rather than thinking of Zn as a specific requirement for entry into cell cycle, we should think of it as a specific requirement for expression of a block of genes during mid-G1 that determine entry into either S phase or a terminal differentiation pathway [2]. The critical point here is that Zn is not simply helping to drive cells into DNA synthesis but rather it is preparing the cells for a subsequent fate, be that cell division or differentiation. In support of this proposal, evidence was provided that, at least in some model systems, entry into differentiation was also dependent on Zn-dependent events in mid-G1. A specific case was differentiation of myoblasts which was inhibited in a low Zn medium; this was accompanied by a reduction in activity and expression of creatine kinase [51]. Another interesting example quoted by Chester and colleagues is the buccal mucosa of rabbits where Zn deficiency suppresses cell differentiation but at the same time drives the cells into S-phase [52]. This has since been confirmed in the rat buccal mucosa and associated with specific changes in keratin processing [53].

2.6. Some Potential Gene Targets of Zn in Cell Cycle Progression

Elucidating the mechanism of action of Zn in cell cycling requires identification of critical gene targets.

2.6.1. Thymidine Kinase

Initial attention focussed on the effects of Zn on induction of thymidine kinase which mediates a salvage pathway for the synthesis of thymidine monophosphate, important for DNA replication [54]. Prasad and Oberleas demonstrated requirements for Zn by thymidine kinase during DNA synthesis in the connective tissue of young rats and in regenerating liver [41]. Rats fed a Zn-free diet for 3 days, prior to partial hepatectomy, had markedly reduced activity of thymidine kinase in their regenerating livers. The addition of Zn, but not other metal ions, to the Zn-deficient enzyme fractions normalized enzyme activity [38; 55]. This latter finding suggested that Zn was acting post-translationally and this is supported by recent structural studies which indicate that Zn is a component of human thymidine kinase [56].

Complicating interpretation of the thymidine kinase studies is strong evidence that Zn also influences transcription of thymidine kinase as mRNA levels for this enzyme were decreased in Zn deficiency; by contrast, expression of histone H3, which also increases in G1, was not inhibited by lack of Zn [57]. When the thymidine kinase gene was placed under the control of the SV40 early promoter, it was insensitive to availability of Zn, indicating a specific effect of Zn on the thymidine kinase gene promoter. Promoter truncation studies mapped the sensitive site to the region from 55 to 83 base pairs 5' to the transcription initiation site [58; 59]. Therefore, one Zn-dependent requirement was the expression of thymidine kinase in late G1. Interestingly, the cells containing the thymidine kinase gene under the control of the SV40 early promoter (where thymidine kinase transcription was now insensitive to Zn), were still

impaired in capacity to enter S phase, indicating that other Zn-dependent gene expression was required for commitment to S phase [58; 59].

2.6.2. Cyclins

Chesters and colleagues concluded that when Zn availability is low, “there is a failure of individual cells to induce the block of enzymes required for DNA synthesis rather than a generalized reduction in activity of enzymes requiring Zn for their catalytic activity or structural integrity”. They recognized that the cell cycle-associated cyclin proteins were also dependent on Zn for their transcription. Levels of cyclin mRNA were strongly decreased in Zn deficient cells [2]. The authors speculated that cyclin expression may be the common factor underlying the requirement for available Zn during both cell replication and differentiation, citing the close temporal relationship between the Zn requirements for cell cycling or differentiation and the induction of gene expression of cyclins in mid-G1. Their studies indicated specific effects of Zn chelation in activated 3T3 cells on expression of cyclins D3, E and B; re-addition of Zn normalized cyclin expression and, in the case of cyclin B, Zn boosted levels above normal, suggesting that Zn may not only restore a defect but actively promote cyclin B expression [2].

2.6.3. Growth Factor Pathways

Many growth factors are Zn-dependent in that they i) contain bound Zn, ii) act synergistically with Zn ions or iii) are suppressed by removal of Zn ions. These include epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin-like growth factor 1 (IGF-I) and nerve growth factor [60-63]. The possibility should be considered that there is a common role for Zn ions in growth factor actions in G1.

Activation of serum-starved 3T3 cells to re-enter cell cycle involves an initial competency-priming step (that can be induced by sequential addition of PDGF for 3 h and EGF for 0.5 h) followed by an IGF-1-dependent step (16 h) [62]. Addition of metal chelators DTPA or EDTA prior to PDGF impaired subsequent DNA synthesis as measured by thymidine incorporation; DNA synthesis was restored by re-addition of Zn but not calcium, iron or cadmium [62]. Total cellular Zn was not different between DTPA-treated and control cells, suggesting the minor labile pool of Zn was being depleted. The first 12 h of the IGF-1 step was most affected by Zn chelation and Zn re-addition. After 12 h, there was no effect of these agents on thymidine incorporation measured at 16 h. It was concluded from this experiment that Zn is not required during the competency-priming step but was required during the later IGF-I stimulatory phase of the cell cycle that is required for entry into S-phase.

That Zn depletion does not affect IGF-1 receptor expression was suggested by the following experiment. Thornton and colleagues compared the effects of serum starvation with that of Zn depletion by DTPA on expression of the IGF-1 receptor in 3T3 cells [64]. A major difference was that while serum-starved cells showed reduction in IGF-1 expression, Zn-depleted cells did not. This was despite Zn depletion reducing the proliferative fraction (S and G2/M phases) of the cell cycle. They concluded that depletion of Zn from culture media inhibits 3T3 cell proliferation independent of IGF-I receptor expression. Since the addition of glutathione to the Zn-depleted media partially restored proliferation, they proposed that the action of Zn may be more related to its antioxidant actions [64]. Others showed that, in the presence of Zn, IGF-1 prefers to bind to its cell membrane IGF receptor rather than to its specific serum binding

proteins [65], implying that Zn acts at the level of IGF-1 binding to its membrane receptor. However, other evidence led Macdonald to conclude that the primary effect of Zn was distal to IGF receptor binding and likely involves one or more steps during IGF-1 receptor-mediated signalling [66].

2.7. Zn Transporters and Cell Cycle Signalling

2.7.1. Enhanced Zn Uptake Radioisotope Studies

Surprisingly, there have been relatively few studies investigating rates of Zn uptake in relation to the cell cycle. One study of note described a 5-fold increase in rate of ^{65}Zn uptake in serum-starved, G1-arrested BHK cells following re-addition of serum to the medium; this increased uptake was associated with increase in diadenosine tetraphosphate concentration [1].

2.7.2. Zn Transporters

A new strategy in probing the role of Zn in cell cycling will be to look for links between Zn transporter expression and specific events in the cell cycle. The discovery over the last two decades of two large families of mammalian Zn transporters (see chapter 8), the dysregulation of which is linked to multiple diseases, is having major impacts on our understanding of the cellular biology of Zn [67]. This will impact also on our understanding of how cells acquire and mobilize Zn during cell cycling. Of immediate primary interest are the Zn transporters so far linked with cancers and perhaps therefore with cell cycle signalling. These include ZIP-4 in pancreatic cancers [68], ZIP-1 in prostate cancer [69] and ZIP-7 in breast cancer [3]. The general picture emerging, with one notable exception, is that cancers are associated with increased intracellular Zn, leading to increased cell cycling (and likely also decreased apoptosis). In this respect, Zn transporters which increase intracellular Zn in the tumor cells (such as ZIP-4 in prostate cancer cells and ZIP-7 in breast cancer cells) should be looked on as growth promoting while those that lower intracellular Zn (such as the efflux transporter ZnT1) might be thought of as growth arresting. The exception to this is prostate cancer, where cancer progression is associated with up to a 10-fold lowering of Zn levels and the influx transporter ZIP-1 behaves as a tumor suppressor [69]. One rationale for this difference between the Zn requirements of prostate cancer versus breast and pancreatic cancer is that the prostate is unique in having very high concentrations of Zn in the normal state [70]. The very high endogenous levels of Zn in normal prostate epithelial cells may be above some threshold conducive to cell cycling. Both very low and high extracellular and intracellular Zn concentrations suppress cell proliferation and are toxic [71; 72]. Therefore, a lowering of Zn concentration in the prostate epithelial cells below a critical threshold may be required to stimulate proliferation. Further lowering of intracellular Zn below the threshold required for induction of cell cycle genes would suppress proliferation. For cancer cells to proliferate, they probably need to maintain their intracellular Zn levels within a narrow window. At this stage, SLC39A4 (ZIP-4) and SLC39A7 (ZIP-7) have been firmly linked to cell cycle signalling via cyclin and tyrosine phosphatase-kinase pathways, respectively, and will be discussed further here (Fig 1 Inset).

Up-regulation of ZIP-4 has been shown to be a tumorigenic mechanism in pancreatic cancer, via increase in cell cycling [73]. In *in vitro* cultures, pancreatic cancer cells expressing high levels of ZIP-4 had increased cell proliferation. The cell

proliferation was Zn-dependent since addition of Zn ions to ZIP-4 over-expressing cancer cell lines increased cell proliferation in a concentration-dependent manner. Furthermore, knockdown of ZIP-4 expression caused reduced proliferation when medium Zn concentration was suboptimal.

As discussed previously, cyclin D plays a critical role in promoting cell proliferation by triggering cell cycle progression from G1 to S phase. Levels of this cyclin are strongly and positively correlated with levels of ZIP-4 gene expression in pancreatic cancer cell lines as shown by ZIP-4 over-expression and knockdown studies. Mechanistic studies suggested the following pathway. Since expression and activity of cAMP response element-binding protein (CREB) is known to be decreased by depletion of intracellular Zn and restored by exogenous Zn ions [74], it was proposed that increased Zn uptake into cells via ZIP-4 leads to increased phosphorylation of CREB [75]. Whether Zn ions directly affect CREB (e.g. via its Zn finger domain) or via affects on NF- κ B or other factor is unclear. Enhanced CREB activity leads to increased transcription of the interleukin (IL)-6 gene via CREB binding to the IL-6 promoter. Increased levels of IL-6, in turn, lead to increased phosphorylation of signal transducer and activator of transcription 3 (STAT3), and enhanced cyclin D1 (and other) gene expression [75].

ZIP-7 has been linked to breast cancer, again via enhanced cell cycling [76]. However, the mechanisms involved with ZIP-7 have at least one important difference from those of ZIP-4. Unlike ZIP-4, ZIP-7 is not found in the plasma membrane but rather localizes to the membranes of the endoplasmic reticulum and/or golgi. ZIP-7 is believed to participate in control of cytosolic Zn levels by allowing the release of Zn ions from these internal stores, in response to certain extracellular stimuli including growth factors [3; 76]. Based on recent studies in mast cells and other types of cell, it is believed that Zn ions released from the endoplasmic reticulum/Golgi constitute a Zn wave which may be calcium-dependent and which inhibits tyrosine phosphatases, thereby enhancing the phosphorylation and activation of tyrosine kinase receptors and leading to a growth stimulus [77].

3. Zn and Cell Death

3.1. Overview of Cell Death Pathways

The period between the 1940s and 1960s brought the recognition that not all cells die traumatically but that there are situations when otherwise healthy cells must die when required as a part of normal organ development and tissue homeostasis. This programmed cell death required the active participation of the cell in its own suicide. When the unique morphological changes of this type of cell death were recognized, the process was renamed apoptosis to distinguish from traumatic membrane rupture and passive cell death that we know as necrosis [78]. In the last decade, other types of cell death have been recognized including pyroptosis and autophagic cell death (Fig 2). These mechanisms have important implications for cell death in inflammation and infection. In considering the common features between the different mechanisms of cell death, Orrenius and colleagues have proposed that “several death executing routines may be activated concomitantly within injured cells, and that one or the other becomes predominant.....dictated by factors as different as energy requirement, signalling molecules or the intensity of a given insult” [79].

3.1.1. Apoptosis (Gene-Directed Cell Death)

Apoptosis (*Greek* “to drop gently like leaves from a tree”) enables the safe removal of cells which are superfluous or damaged, without affecting neighbouring cells. Dysregulation of apoptosis is central to the pathogenesis of a number of diseases, being too low in cancer and some autoimmune diseases and too high in neurodegenerative disorders and diabetes mellitus [80]. The hope is that selectively stimulating or suppressing apoptotic signalling pathways will provide new therapeutic opportunities in disease.

Apoptosis is an active process requiring energy and, in some cases, new gene expression. It is characterized by cell shrinkage, condensation of the cytoplasm and nucleus, DNA fragmentation, changes in the membrane phospholipid composition and subsequent fragmentation into apoptotic bodies which are cleared by macrophage-mediated efferocytosis or lost via shedding into body cavities [78]. The non-involvement of plasma membrane rupture ensures that the cells dying by apoptosis do not release potentially toxic or pro-inflammatory factors. Failure of efferocytosis may lead to accumulation of apoptotic bodies in the tissues with eventual lysis and release of contents, thereby creating a pro-inflammatory stimulus (Fig 2).

In addition to physiological roles of apoptosis in development and tissue homeostasis, apoptosis also deals with the removal of damaged cells (e.g. by oxidative stress or DNA damage). Oxidants such as superoxide anion, hydroxyl radical and peroxynitrite are effective inducers of apoptosis, while some classical anti-apoptotic agents (e.g. Bcl-2) have turned out to be effective anti-oxidants [81; 82]. This has led to the suggestion that apoptosis may have evolved primarily to rid the body of oxidatively-damaged cells [83]. In response to DNA damage and some other cellular stresses, p53 turnover is decreased and its levels increase, promoting the transcription of a number of genes involved in DNA repair, apoptosis and cell cycling. Cells in cycle arrest at G1/S and may proceed to apoptotic cell death [84].

Two major pro-apoptotic signalling pathways have been described, both involving a novel family of aspartate-specific proteases called caspases [85]. Caspases, of which there are more than 15 members, pre-exist as zymogens and are activated either by self-aggregation or by proteolytic processing by other caspases or related enzymes.

One of the two major apoptosis signalling pathways is known as the extrinsic pathway because the initiating death signals are primarily external, such as the ligation of Fas, tumour necrosis factor and other receptors in the plasma membrane. The accompanying receptor clustering leads to formation of a platform known as death inducing signalling complex (DISC) that generates active caspase-8, an upstream caspase in apoptosis [86]. Caspase-8, in turn, activates the major effector caspase, caspase-3.

The second (or intrinsic) pathway is triggered by changes at the level of the mitochondria, including release into the cytosol of cytochrome c, and resulting in activation of the upstream caspase-9 on a platform known as the apoptosome [87]. Pro-caspase-9 is activated by formation of a complex with apoptosis protease-activating factor (Apaf-1) and with cytochrome c. Active caspase-9 also causes activation of caspase-3, ensuring that the extrinsic and intrinsic pathways converge at the level of the downstream caspases. This intrinsic pathway is tightly regulated by two families of regulators, one pro-apoptotic (e.g. Bax) and the other anti-apoptotic (e.g. Bcl-2) [88]. Caspases are also subject to regulation by a family of endogenous cellular proteins called the Inhibitors of Apoptosis proteins (IAPs), the best characterized being the X-

linked XIAP [89]. These suppress caspase activity by binding to and inhibiting regulatory or catalytic parts of the active caspase molecule or its zymogen, or by targeting them for degradation by proteasomes.

Cleavage of critical protein targets by the downstream or executioner caspases leads to events associated with the morphological changes such as cell shrinkage and the condensation of the chromatin; several hundred cellular caspase substrates have been identified [90]. Since these include cell cycle regulators such as p21 Waf1/Cip1[91], caspases may play a pivotal role in cell cycling and cell survival pathways. Indeed, such a pivotal role has been suggested recently for caspase-2 [92] which becomes pro-apoptotic when co-expressed in cells with cyclin D3, but not in the absence of this cyclin [93]. Caspase-2 is involved also in the cell cycle G2/M DNA damage check-point [94].

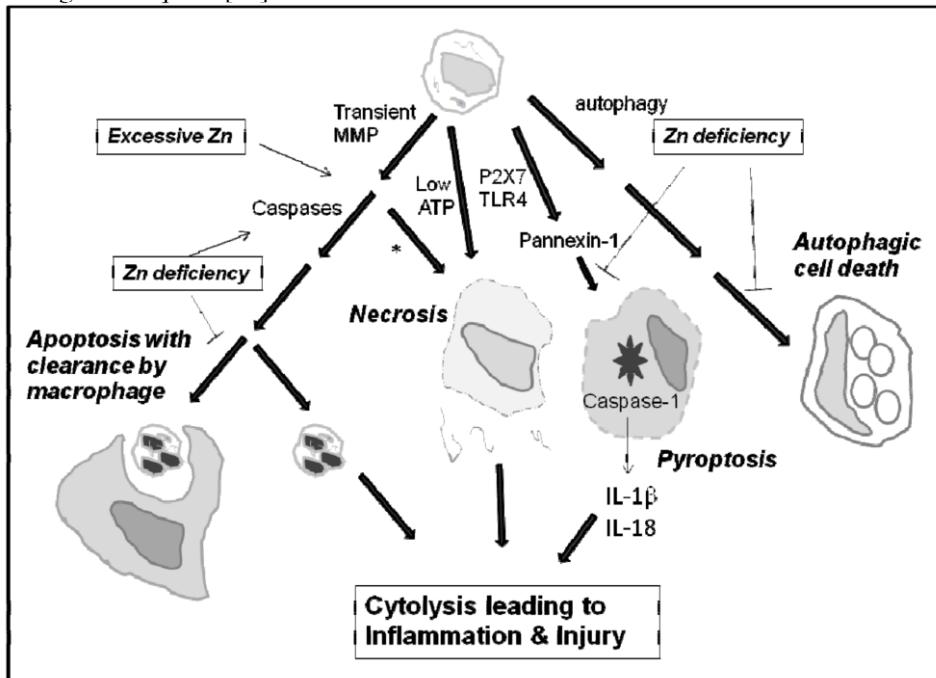


Figure 2. Roles of Zn in cell death. Pathways leading to the four major mechanisms of cell death are shown (from left to right: apoptosis, necrosis, pyroptosis and autophagic cell death). Normally, only necrosis and pyroptosis lead to cell lysis and inflammation. In apoptosis, early events include transient opening of mitochondrial pores to release cytochrome c and facilitate caspase activation. Apoptotic bodies containing condensed chromatin and other cellular components are cleared by phagocytic cells; if efferocytosis is impaired, as occurs in Zn deficiency, the apoptotic bodies may undergo secondary necrosis and provoke inflammation. Both excessive and insufficient Zn can trigger apoptosis. In necrosis, MMP (mitochondrial membrane permeability) is sustained and ATP levels decline leading to membrane rupture. Apoptosing cells can also succumb to a necrotic death if their caspases are blocked (*). In pyroptosis, signals from TLR4 and P2X7 receptors, coupled to the pannexin-1 membrane channel, lead to the formation of the inflammasome (depicted by the star-shaped structure) which activates caspase-1 resulting in processing and release of IL-1-like cytokines that drive systemic inflammation. Zn deficiency is thought to block pyroptosis at the level of pannexin-1. In autophagic cell death, formation of membranes around organelles destined for recycling precedes intense vacuolization and cell death. Zn deficiency prevents autophagic death.

3.1.2. Necrosis (*Traumatic Cell Death*)

Necrosis is the way cells die when severely damaged or sufficiently depleted of energy to interfere with membrane permeability or calcium ion homeostasis. While necrosis is considered passive cell death, recent studies indicate some pathways in common with apoptosis. Both processes involve the opening of a pore in the inner mitochondrial membrane known as the mitochondrial permeability transition pore, resulting in increased mitochondrial membrane permeability (MMP) [95]. The difference between necrosis and apoptosis is that with necrosis the opening of the pore is sustained, depleting ATP and compromising plasma membrane integrity, while with apoptosis the pore opens transiently, allowing maintenance of ATP levels but release of cytochrome c and other factors that promote activation of the caspases [96]. Apoptosis is the default mechanism of cell death and the preferred way of cells dying in the body. In models of cell damage-induced apoptosis, if apoptosis is blocked downstream of the mitochondrial changes (e.g. by caspase-inhibitors), the damaged cell is still likely to die, but now by necrosis rather than apoptosis (Fig 2). For agents to be fully cytoprotective, they may need to act on pathways upstream of the mitochondria or by blocking MMP [97]. The powerful cell survival factor Bcl-2 has been considered to be an MMP blocker and therefore both anti-apoptotic and anti-necrotic [98].

3.1.3. Autophagic Cell Death (*Cytoplasmic Cell Death or Type II Cell Death*)

Autophagy (*Greek “self-eating”*) is a tightly-regulated catabolic process that enables cells to recycle their organelles and long-lived proteins. Common triggers include hormones or starvation [99]. For example, when starved of essential amino acids, hepatocytes utilize autophagy to replenish stocks of these amino acids required for gluconeogenesis [100]. The process involves the formation around organelles of a double membrane derived from the endoplasmic reticulum. The resulting structure is known as an autophagosome and this fuses with lysosomes which provide the digestive enzymes to begin the recycling [101].

If starvation persists, autophagy can proceed beyond normal recycling to a unique mechanism of cell death known as autophagic cell death (Fig 2). This mechanism of cell death was recognized in the late 1960s to be another type of programmed cell death and shown to be important not only for deleting starved cells but also for normal physiological processes such as tissue homeostasis, differentiation, metamorphosis of insects and neuronal development [102; 103]. Less is known about its involvement in pathogenesis but it would appear to be a feature, at least, of neurodegenerative disease [103]. Morphologically, autophagic cell death is distinguished from apoptosis by absence of chromatin condensation and presence of massive vacuolization of the cytoplasm [104]. This vacuolization is one manifestation of the intense degradation of cytoplasmic organelles and long-lived proteins that is underway. The signalling pathways in autophagic cell death are poorly understood. Interestingly, p53 may promote apoptosis or autophagy, depending on whether it is primarily acting in the cytoplasm or nucleus, respectively [105].

3.1.4. Pyroptosis (*Inflammatory Cell Death*)

A novel form of cell death was discovered recently as a consequence of studies into innate immunity and the production of the pro-inflammatory cytokine IL-1 β . It has been named pyroptosis (*Greek “going down in flames”*) or inflammatory cell death

[106]. This form of cell death, particularly seen in infection with intracellular pathogens and thought to be part of the anti-microbial response, involves some features of necrosis such as cellular swelling, osmotic lysis and plasma membrane rupture. Also as in necrosis, release of the cell contents may provoke an inflammatory response. The process however differs from necrosis, and is dependent on caspase-1, but not the apoptotic caspases. Little is known about the mechanisms leading specifically to cell death, other than the important role of an intracellular scaffolding known as the inflammasome, a structure which is formed in cells undergoing stress or infection as a mediator of innate immunity (Fig 2, star-shaped structure) [107]. The inflammasome is somewhat analogous to the apoptosome, except that it generates active caspase-1 rather than active caspase-3.

The inflammasome assembly is triggered as a result of two signalling events (reviewed in [107; 108]). Activation of membrane toll-like receptor 4 (TLR4) by lipopolysaccharide or other microbial components is an early step in the innate immune response to pathogens. One of the consequences is increased transcription of the IL-1 family of genes (IL-1 β , IL-18, IL-33). However, these cytokines are not released until a second signal is generated via P2X7 receptors in response to ATP and other danger signals. Exactly how the cytokines are released is currently being studied but caspase-1, pannexin-1 and cell death/cytolysis have been implicated [109-111]. Caspase-1, which is activated on the inflammasome following P2X7 signalling, cleaves pro-IL-1 to its mature form, IL-1 β . Pannexin-1 is a membrane protein which, following ligation of P2X7 receptors by ATP, forms a membrane channel that is permeable to IL-1 β [112]. In the process, the inflammasome-containing cell may lyse providing another mechanism for IL-1 β release [111].

3.2. Zinc and Cell Death

Much of what we know about effects of Zn on cell death relate to its effects on apoptosis. However, there is increasing evidence that it affects at least some of the other cell death processes, as well.

3.2.1. Zn and Apoptosis: General Comments

The literature on Zn and apoptosis can be conveniently broken up into three themes:- the anti-apoptotic effects of Zn, the pro-apoptotic effects of Zn and the evidence that Zn homeostasis is substantially disturbed in apoptosis. A number of detailed reviews on the earlier papers relating Zn and apoptosis can be found elsewhere [113-115].

Not long after the identification of apoptosis by Kerr, Wyllie and Currie as a novel mechanism of cell death [116], Margaret Elmes, a colleague of Andrew Wyllie, described the dramatic increase in apoptosis *in vivo* in several tissues of Zn deficient rodents, including the intestinal epithelium, skin, and testis [117; 118]. Subsequent studies by ourselves and a large number of other groups, mainly in *in vitro* model systems, strongly suggested that the increase in rate of tissue apoptosis in Zn deficiency was a direct consequence of lowering of Zn levels in the affected tissue cells [113; 114]. However, before describing some of this evidence and the insights into the anti-apoptotic mechanisms of Zn, three pertinent points need to be mentioned.

Firstly, some of the effects of Zn deficiency may not be direct but mediated via a stress response induced by *in vivo* deprivation of Zn. Studies by Pamela Fraker (Michigan State University) have had a major impact on our understanding of how Zn

deficiency-induced apoptosis disturbs early B and T cell development [119; 120]. Some of the effects of Zn deprivation (e.g. in the thymus and bone marrow) are not direct effects on the lymphocytes but mediated by changes that Zn deficiency causes in the hypothalamus-adrenal-pituitary axis, resulting in increased circulating glucocorticoids, which themselves are powerful apoptogens for immature lymphoid cells [120].

Secondly, an increased frequency of apoptotic cells in a tissue does not necessarily mean that the rate of induction of apoptosis has increased. *In vivo*, apoptotic cells are rapidly cleared by macrophage-mediated efferocytosis or engulfment by other types of cell [121]. However, if the clearance rate decreases (e.g. as in the diseased lungs of patients where alveolar macrophage functions are impaired [122]) there is a readily-detectable increase in frequency of apoptotic cells. This has important implications for disease since failure of apoptotic epithelial cells to be cleared may result in their secondary necrosis and consequent release of pro-inflammatory factors. Elegant recent studies by Joshi and colleagues have shown that in lungs of rodents exposed to stresses such as chronic alcohol exposure and HIV infection, there are abnormally low Zn levels in the lungs and impaired alveolar macrophage phagocytosis, which could be entirely corrected by Zn supplements [123; 124].

The third point concerning *in vivo* Zn deficiency is that cycling cells (e.g. in the intestinal epithelium and testes) appear to be especially sensitive to Zn depletion-induced apoptosis [114; 118]. There is likely to be close interplay and feedback between cell cycle and cell death signalling pathways that can be affected by Zn depletion. For example, caspase-3 dependent cleavage of the cell cycle regulator p21^{waf1/cip1} occurs immediately following treatment with the membrane-permeable Zn chelator N,N,N',N'-tetrakis-(2-pyridyl-methyl) ethylenediamine (TPEN) [91]. Loss of p21 leads to a dramatic induction of cdk2 activity which may result in premature entry of the cells into S-phase and apoptotic cell death [125].

While bearing in mind that some *in vivo* effects of Zn depletion on apoptosis *in vivo* may be indirect, there is substantial *in vitro* evidence showing that growth of many types of cell in Zn deficient medium or treatment of cells with relatively specific Zn chelators such as TPEN directly switches on the caspase cascade and other pro-apoptotic signalling pathways within the cells about to apoptose [126; 127]. The mechanism of Zn deficiency-induced cell death *in vitro* and *in vivo* has the classic features of apoptosis, including the major morphological features of apoptosis, DNA fragmentation, chromatin condensation, apoptotic body formation and caspase-3 activation [114; 128]. Relatively small changes in labile Zn were able to cause large changes in susceptibility of cells to apoptosis [126]. Small decreases in intracellular Zn may themselves induce little apoptosis but rather increase susceptibility of cells and tissues to the effects of toxins. In support of this critical role for Zn in toxin resistance, supplementing cells with exogenous Zn decreases the susceptibility of cells and tissues to spontaneous or toxin-induced apoptosis [129-132]. Summarizing these studies, a small drop in intracellular Zn renders cells more susceptible to death by subsequent exposure to toxin, while a larger drop in intracellular Zn is sufficient to initiate the apoptosis cascade.

3.2.2. Zn, Caspases and Inhibitors of Apoptosis Proteins (IAPs)

Initially, the focus was on suppressive effects of Zn on apoptotic endonucleases but evidence that Zn was anti-apoptotic at concentrations below that required to block the

endonucleases and that Zn was capable of blocking apoptosis in nuclei-free cytoplasts indicated that the primary target of Zn was not an endonuclease [114]. Lazebnik and colleagues first defined the important role of a cytosolic caspase in mammalian cell apoptosis, and noted that either the caspase or an upstream effector was Zn-sensitive [133]. Some, but not all, studies have shown direct inhibitory effects of Zn on active caspase-3 in semi-purified models [134; 135]. Our own studies using a cell-free model of apoptosis have shown partial inhibitory effects of Zn on caspase-3 catalytic activity but more pronounced effects, at lower Zn concentrations, on a step(s) involved in the activation of procaspase-3 [114]. Other reports have confirmed an inhibitory effect of Zn on pro-caspase-3 activation [136; 137]. Zn also blocks other potential downstream and upstream caspases including caspase-6 and caspase-9, respectively [138].

Our studies revealed a short lag (~ 30 min) between decline in intracellular Zn induced by TPEN and increase in cytosolic caspase-3 activity [129]. One possibility is that a drop in Zn in the environment of procaspase-3 triggers active caspase-3 formation, either by removing inhibitory Zn or by inactivating a Zn-dependent inhibitor of caspase-3 formation. It could also be that the direct effect of Zn depletion is on an upstream caspase or other upstream pro-apoptotic signalling molecule. A decline in intracellular Zn below a critical threshold level may not only trigger pathways leading to caspase activation but may also facilitate the process by which the caspases, themselves, are activated. Further evidence that Zn inhibits at the level of procaspase-3/caspase-3 comes from studies with a compound PAC-1 (procaspase activating compound) that was identified from screening studies as a direct activator of procaspase-3 in cell-free systems and an inducer of apoptotic cell death in intact cells and tumors [139]. Using a purified system in which autoactivation of recombinant procaspase-3 to the active caspase was strongly inhibited by the presence of Zn in the buffer, it was shown that PAC-1 bound Zn and relieved the inhibition of procaspase-3, allowing active caspase-3 activity to generate; PAC-1 had no further enhancing activity when the buffer was Zn-free [140; 141]. At least one study has shown that Zn (albeit at pharmacological concentrations) can block the mitochondrial pore through which cytochrome c is released [142]. This needs further investigation, particular looking at the effects of cellular Zn deprivation on the pore formation.

Our group has become interested in similarities between the anti-apoptotic effects of Zn ions and those of the Inhibitor of Apoptosis (IAP) proteins, including X-linked inhibitor of apoptosis (XIAP). IAPs are a family of endogenous cellular proteins which, like Zn ions, have the potential to suppress both the catalytic activity of caspases and the steps leading to their formation [89]. It is interesting therefore that most IAPs contain two distinct types of Zn finger motifs: BIR domains which bind to and inactivate (pro)caspases and a C-terminal Zn RING finger which mediates caspase ubiquitylation leading to their proteasomal degradation. The Zn in RING fingers appears to be relatively labile (especially in the presence of oxidants). The RING finger of a viral IAP was highly Zn-dependent, and, importantly, responded further to addition of μ M concentrations of exogenous Zn, by increasing E3 ligase activity, suggesting the potential of this family of caspase-inhibitory proteins to be regulated by a cellular Zn flux [143; 144]. These findings raise the possibility that at least a part of the inhibitory effect of labile Zn ions on apoptosis is mediated via Zn fingers of IAPs. This hypothesis is supported by recent findings that TPEN induced a rapid proteasome-independent and caspase-independent degradation of XIAP in human cancer lines [71]. In view of the well-known auxillary role that serine proteases play in apoptosis [145] and since the serine protease inhibitor, pefabloc, prevented the loss of XIAP in TPEN-

treated cells [71] it was suggested by Makhov and colleagues that Zn depletion triggers serine-protease-induced cleavage of XIAP. TPEN may act by removing inhibitory Zn from the serine protease or from XIAP (perhaps making the cleavage site accessible to the protease). Whether degradation of XIAP is responsible for Zn deficiency-induced activation of caspase-3 needs now to be determined.

3.2.3. Zn, Oxidative Stress and Apoptosis

The above studies have considered direct effects of Zn depletion on the apoptotic regulatory molecules. An alternative view is that Zn deficiency does not itself turn on caspases or upstream effectors but rather damages the cell in such a way that the cell responds by turning on its caspases and proceeding to death. In line with this view, is the well-known effects of Zn deficiency on oxidative damage. Studies by our laboratory and others have shown that Zn deficiency increases the susceptibility of cells *in vitro* to a variety of oxidants, as measured by increased lipid peroxidation [129]. Animal studies have linked Zn deficiency with enhanced rates of oxidative damage while Zn supplementation protected against oxidative damage, *in vivo* and anti-oxidants protected against Zn-deficiency induced lesions (reviewed in [114]).

The real answer to how Zn deficiency triggers the apoptotic cascade may be a mixture of i) its direct effects on apoptosis signalling molecules and ii) its capacity to protect cells from damage. By this reasoning, Zn limits the extent of damage induced by oxyradicals and thereby suppresses the need for the cell to activate caspases and die. At the same time by protecting an essential sulphhydryl(s) at the active site of caspases, it is able to directly and reversibly suppress the caspase cascade, as well.

3.2.4. Zn and P53

As described earlier, the p53 tumor suppressor gene protein influences both cell cycle and cell death regulation. It also contains a functional Zn atom which binds to three strategically-located cysteines in p53. This Zn is functionally important as i) it protects p53 from oxidation, ii) it is located near its DNA binding domain and is required for DNA binding of p53 and transcriptional activation, iii) it ensures proper p53 folding since both excessively low and high Zn cause p53 to misfold by distinct mechanisms resulting in impaired function; and iv), when lost, it appears to have a similar impact on p53 function as does p53 mutation in cancer [146]. Ra and colleagues have shown a strict requirement for p53 in TPEN-induced neuronal apoptosis and concluded that removal of Zn from p53 increases both its stability and activity [147; 148]. Two other very interesting observations emerged from this study. Firstly, TPEN induced activation of caspase-11 in a p53-dependent manner. Caspase-11 has been little studied from the point of view of apoptosis but is known as an activator of caspase-1 and a mediator of septic shock [149]. Secondly, cytochrome c release from mitochondria and caspase-3 activation also occurred downstream of p53 and caspase-11. It would appear that the caspase-11 mediated activation of caspase-1 and caspase-3 occur by distinct pathways [150]. Clearly, the mechanism described by Ra et al in neuronal cells is distinct from the rapid TPEN-induced induction of caspase-3 activity (within 2h) that we and others have found in some other types of cell [151].

3.2.5. Pro-Apoptotic Roles of Zn

While a large body of evidence, *in vitro* and *in vivo*, indicates that Zn is a physiological suppressor of apoptosis, there are also many published studies showing the opposite, that Zn is also a potent inducer of apoptosis (and/or necrosis) [152-155]. This phenomenon has been well-studied in acute ischaemic or traumatic brain injury where an increase in the level of labile Zn in astrocytes and neurons is considered one of the major causes of death of these cells. It has been proposed that the Zn-induced toxicity is a consequence of Zn release from internal stores due to oxidative/nitrosative stress and can be suppressed *in vivo* by agents such as nitric oxide synthase inhibitors [156]. There are several plausible explanations for these opposing effects of Zn.

Firstly, it may be dependent on the type of cell or the tissue in which the cell is located. Many of the studies showing pro-apoptotic roles of Zn have involved neuronal cells. Perhaps there is something special about these cells or their extracellular milieu which makes them sensitive to Zn ions. However, non-neuronal cells can also die due to Zn toxicity (e.g. in mouse dendritic cells treated with low concentrations of Zn there was a rise in ceramide concentration, a potent inducer of apoptosis, preceding morphological changes of apoptosis; in acid sphingomyelinase null cells, which were unable to produce ceramide, much higher concentrations of Zn were required for toxicity in the null cells [157]). In another study, a low concentration (10uM) of extracellular Zn induced apoptosis in human ductal adenocarcinoma cell lines, while normal human pancreatic islet cells were unaffected by a 50-fold higher concentration of Zn [158].

Whether a cell is protected or killed by a rise in intracellular Zn may also be a question of the levels of intracellular Zn reached and in what compartment. In this scenario, there is a narrow window of intracellular Zn concentrations that permit cell survival:- intracellular Zn concentrations below the window (as might occur in Zn-deficient cells) while intracellular Zn concentrations above the window (as might occur in cells exposed to toxic concentrations of Zn) would also trigger apoptosis. The cellular compartment in which Zn content rises might be important (e.g. intra-mitochondrial Zn may be especially toxic [159]). It is pertinent to ask whether the window of intracellular Zn concentrations that permit cells to cycle through mitosis is the same window as that permitting cells to avoid death by apoptosis.

Thirdly, and perhaps more speculatively, whether cytosolic Zn levels suppress or promote apoptosis may depend on the presence or absence of specific Zn transporters and Zn muffler proteins like thionein. Interestingly, neuronal Zn excitotoxicity can be blunted by a preconditioning period in which sub-toxic concentrations of labile Zn are induced by release of Zn from metallothionein and this Zn release then triggers Zn-dependent events that mediate neuroprotection [160]. The relevance of this phenomenon to Zn-mediated regulation of cell death mechanisms in other tissues warrants further investigation.

Some of these issues have begun to be addressed in a rat model of epileptic seizures [161]. After seizures induced by kainic acid, inter-neurones died while CA3 pyramidal cells survived. Measurement of intracellular labile Zn using Zn fluorophore revealed a 2-3 fold higher Zn level in the inter-neurones (estimated to be 600nM compared to 250 nM in the pyramidal cells). Chelating the Zn or feeding the rats a low Zn diet increased survival of the inter-neurones while decreasing survival of pyramidal cells. The authors have proposed that the low Zn levels in the pyramidal cells are

cytoprotective by enhancing anti-apoptotic pathways whereas the higher Zn level in the inter-neurones interferes with mitochondrial function and are cytotoxic.

3.2.6. Changes in Zn Homeostasis during Apoptosis

Studies with a variety of Zn fluorophores have shown that early in apoptotic cell death there is a dramatic rise in intracellular labile Zn, suggesting a major change in Zn homeostasis [4; 5; 162]. Whether this rise in Zn is entirely secondary to changes occurring in the apoptotic cell or a factor in the subsequent apoptosis (similar to Zn-mediated apoptosis described above) is not clear. It might be informative to look at the effects of low doses of Zn chelators in classical apoptosis induction models (e.g. treatment of cells with staurosporine or etoposide). Although we have not deliberately tested this in our own models, we have only ever seen no effect or enhancement of apoptosis when a combination of a toxin (staurosporine, colchicine, peroxynitrite) was used in combination with a wide range of TPEN concentrations. Another question concerns the source of Zn. Early speculation was that the Zn was released from metalloproteins as a result of an increase in oxidation state of the cytoplasm early in apoptosis [4]. However, it may represent Zn released from internal stores (e.g. endoplasmatic reticulum (ER) and golgi). Now that Zn transporters mediating ER/Golgi influx and efflux have been identified, it would be worth revisiting this phenomenon. For example would knockdown of ZIP-7 prevent the Zn rise early in apoptosis? Another intriguing question is, given the strong inhibitory effects of Zn ions on caspases and some other pro-apoptotic signalling pathways, how can apoptosis proceed following the early Zn mobilization. While we have not examined this in detail, we have observed a sequestration of Zn ions (detected by Zinquin), from active caspase-3 (detected by antibody), in dual-labelled cells undergoing early stages of apoptosis (Zalewski Unpublished observation). It may be that caspases and other factors required for apoptosis are in distinct compartments from that of the mobilized Zn ions.

3.2.7. Spatial Relationships between Zn and Apoptotic Regulators

We need to better define the spatial and/or temporal relationships between (i) the mobilized pool(s) of cytosolic Zn in all cells undergoing early apoptosis, (ii) the intracellular Zn pools involved in disease-associated neurotoxicity and seemingly responsible for the ensuing apoptosis of neurons and other brain cells and (iii) the intracellular Zn pools that mediate anti-apoptotic effects in most types of cell. Each of these pools is readily accessible to Zn fluorophores and Zn chelators such as TPEN. Are these pools of Zn distinct in some way or is it more an issue of when the Zn mobilization occurs in relation to other events in the damaged cell that determines the influence of Zn on the fate of the cell? One could imagine that, as with the mitotic G1 restriction point, passage of a critical check-point early in apoptosis renders the subsequent events refractory to Zn ions or perhaps even enhance the downstream processes.

Polarized cells offer a better opportunity to study spatial relationships between intracellular pools of stainable Zn and apoptotic regulator factors. In our laboratory, we have utilized the columnar epithelial cells lining the bronchioles, trachea and nasal cavity (airway epithelial cells, AEC) as polarized cell models to study Zn-regulated, oxidant-triggered, apoptosis [163]. AEC have a ciliated plasma membrane facing the airway lumen. Beneath the cilia are abundant mitochondria for provision of energy for

ciliary beating. The major oxidative threat to these cells comes from the air and pollutants within the lumen and from the respiring mitochondria which leak oxy-radicals. This same apical region was also rich in both pro-caspase-3 and labile Zn (the latter within cytoplasmic vesicles) [130; 163]. Procaspsase-3 is strategically-placed to trigger apoptosis should mitochondrial- or luminal-derived oxidants become a threat. Zn is also strategically placed to protect apical membranes from oxidation and to maintain procaspsase-3 in an inactive state until required. In a mouse model of asthma, labile apical Zn was lost, procaspsase-3 diffused throughout the cytoplasm and epithelial apoptosis became prominent; nutritional Zn restriction further increased the apoptosis [164]. These changes may be a factor in the fragility of the airway epithelium in asthma. It will be interesting to determine whether Zn is similarly localized near procaspsase-3 in other polarized cell models.

In summary (Fig 2), Zn deficiency promotes apoptotic cell death (activating p53, caspases and other pro-apoptotic effectors) and likely impairs the clearance of apoptotic corpses from tissues. Under some circumstances that are not properly understood, Zn can trigger apoptotic cell death and cause pathology (e.g. in the brain). Factors such as cell type, compartment of cell in which Zn accumulates (e.g. mitochondria) and concentrations of intracellular Zn reached may influence whether a cell undergoes Zn-induced apoptosis.

3.2.8. Zn and Necrosis

While we know a lot about Zn and apoptosis, we know relatively little about Zn and necrosis or other cell death mechanisms. There are examples (e.g. T cell leukaemic Molt-3 cells) and TPEN-treated human renal cell carcinoma cell lines (lacking caspases-3, -7, -8 and -10) where Zn deficient cells died by necrosis rather than apoptosis [165; 166]. This, however, does not prove that Zn is also anti-necrotic. If cells are damaged or otherwise significantly stressed and if their pathway to apoptosis is blocked (as in the caspase-defective renal carcinoma line or if ATP levels are sufficiently low), then death may simply switch from apoptosis to necrosis.

3.2.9. Zn and Autophagy

Trafficking of Zn vesicles and protein complexes along axons or dendrites in neurons suggested that a scavenging pathway for these Zn structures may exist in neurons. This led to experiments to test the hypothesis that depleting intracellular Zn in neurons would disturb neurite integrity and dynamics. Zn chelators caused degeneration of axons and dendrite in a retrograde manner but left the cell body intact. The effect was due to chelation of Zn, but not other metals and was accompanied by a decrease in ATP within the neurites. Moreover, neurite degeneration was caused by a decrease in neuritic ATP levels as it could be reversed by addition of nicotinamide adenine dinucleotide and it was partially blocked by 3-methyladenine, an inhibitor of autophagy. The authors concluded that a fall in neuronal Zn levels led to depletion of ATP in the neurites and their autophagic degeneration, although induction of autophagic cell death of the neurons was not reached [167].

An opposite finding was made in astrocytes damaged by oxidants and in ethambutol-treated retinal cell cultures (discussed in detail in [100]). Chelation of intracellular Zn by TPEN mimicked autophagy inhibitors (3-methyladenine, bafilomycin-1) in suppressing autophagic vacuolation and cell death while addition of Zn increased the number of autophagic vacuoles. During autophagy, levels of labile Zn,

as detected by FluoZin-3, increased in the vacuoles as well as in the cytosol and nuclei, somewhat analogous to the increase in labile Zn seen in cells dying of apoptosis. TPEN blocked the increase in labile Zn and the lysosomal activation and lysosomal membrane permeabilization. Of interest, inhibition of autophagy blocked the rise in labile Zn levels, suggesting that this rise is a consequence, rather than a cause, of the autophagy [168]. Subsequent studies with metallothionein-3 null mice have implicated metallothionein-3 in the cell death, perhaps by releasing toxic levels of Zn during oxidative stress [169].

A similar conclusion, that Zn mediates autophagy, has also been reported for tamoxifen-induced vacuolization and autophagic cell death in MCF-7 cells. As in the previous studies, levels of labile Zn increased in the acidic autophagic vacuoles and TPEN blocked both the vacuolization and cell death while exogenous Zn strongly increased them. Effects of TPEN and exogenous Zn were associated with increase and decrease, respectively, in ERK phosphorylation. Another critical event in the cell death was the increase in lysosomal membrane permeability and subsequent release of cathepsin D into the cytosol, both processes blocked by the Zn chelator. Since cathepsin inhibitors blocked the cell death, cathepsin D may be the effector of autophagic cell death in this model. The antioxidant N-acetyl-L-cysteine also partially suppressed the increase in labile Zn levels, cathepsin D release into the cytosol and cell death [6]. The following scenario was suggested: oxidative stress leads to an increase in labile Zn associated with formation of autophagic vacuoles, release of cathepsin D into the cytosol and subsequent cell death.

In summary, Zn deficiency appears to prevent autophagic cell death.

3.2.10. Zn and Pyroptosis

Since Zn ions are both anti-inflammatory and cell survival-promoting, one wonders whether Zn is also able to modulate the inflammatory cell death processes, such as pyroptosis. Indeed, there are many similarities between the apoptosome platform which activates caspase-3 and which is suppressed by Zn, and the inflammasome platform which activates caspase-1. Both involve the formation of a multi-protein complex held together by specific adaptor molecules and interacting with the CARD domain of caspases and BIR domains of inhibitory IAPs. To my knowledge, there is no strong evidence as yet that Zn directly inhibits the inflammasome and caspase-1 generation but, with some exceptions (see below) this has yet to be properly studied.

In one study [170], mouse peritoneal macrophages were primed for 2h with 1 µg/mL lipopolysaccharide. They were then treated with TPEN (50 µM, 15 min) to chelate intracellular Zn prior to 10 min incubation with 5 mM ATP. TPEN abolished the release of IL-1β in response to ATP and this was reversible by addition of exogenous Zn ions. The effect was attributed to inhibition of pro-IL-1β processing resulting from an inhibition of caspase-1 activation, as shown by western blotting with antibody to the active p10 subunit of caspase-1. Specificity for IL-1β was shown by lack of effect of TPEN on IL-6 release. Effects of TPEN on IL-1β release occurred prior to ATP-induced cell death as measured by lactate dehydrogenase release and were associated with an inhibition of pannexin-1. Therefore, the primary effects of Zn chelation in blocking IL-1β release were due to blocking of the pannexin-1 channel rather than via loss of membrane integrity. However, ATP-treated cells did eventually die (after a further 25 min incubation); death was caspase-1 dependent (typical of pyroptosis) and this cell death was completely blocked by TPEN, in agreement with the

inhibition of caspase-1 activation by TPEN. In a cell-free model of inflammasome assembly and caspase-1 activation, TPEN had no effect. The authors therefore proposed an action of Zn chelation upstream of the inflammasome and likely on pannexin-1. This was confirmed by showing TPEN also blocked ATP-induced ethidium dye uptake via the pannexin-1 channel [170]. This finding appeared to be contradicted by other studies showing that submicromolar concentrations of Zn (and copper) ions actually blocked P2X7 receptor-mediated pannexin 1 channel opening as detected by blocking entry through the channel of propidium dyes; however, exogenous Zn ions may be having a direct effect on the ectodomain of P2X7 receptors distinct from endogenous Zn facilitating the pannexin-1 ion channel, presumably at the cytoplasmic side of the membrane [171; 172].

In summary, current evidence is that Zn deficiency prevents pyroptosis by blocking pannexin-1 but this requires confirmation in other models.

4. Conclusion and Perspectives

The capacity of endogenous and exogenous Zn ions to regulate distinct processes in both cell cycling and cell death pathways ensures that dynamic Zn fluxes play a pivotal role in both tissue homeostasis and development. It is probably no coincidence that many growth factors contain Zn or require its presence for their mitogenic activities while many cell death regulating factors (caspases, p53, XIAP etc) are held in check by Zn. Nutritional zinc deficiency or abnormalities in Zn homeostasis such as occurs in tumors, inflammation and ischaemia will facilitate pathogenic mechanisms and we need to explore this further in the context of specific diseases.

Amongst the most important of challenges in the next decade of Zn biomedical research will be to identify the expression and roles of the two families of Zn transporters at specific stages of the cell cycle. The finding of up-regulation of two ZIP family Zn transporters in cancers and the demonstration that forced up-regulation or down-regulation of these transporters in cancer cell lines stimulates and inhibits growth, respectively, fits nicely with the concept that a rise in cytosolic Zn ions in mid-G1 is a pre-requisite for subsequent passage through the cell cycle. What is the Zn transporter(s) that mediates the critical Zn flux in mid-G1 required for new gene expression? To what extent is the Zn flux due to changes in plasma membrane Zn transporter expression as opposed to internal organelle membrane Zn transporters and is a release of Zn from the golgi or endoplasmic reticulum functionally equivalent to an influx of Zn across the plasma membrane with respect to control of mitosis? Since so many studies have shown dramatic effects on cell cycling by simply chelating extracellular Zn, presumably the internal stores of Zn are limited and/or need constant topping up from the extracellular pools, although it could be argued that the metal chelators used in these studies are preventing an influx of calcium ions which might be required for release of Zn from internal organelles. The transporter studies have drawn attention to two candidate proximal down-stream targets of a Zn wave in G1, tyrosine phosphatases and CREB. This should stimulate further experimentation.

Equally important is understanding the roles of Zn transporters and other Zn binding proteins in cells undergoing regulated cell death. The large fluxes of Zn that we can easily see in apoptotic and autophagic cell death by Zn fluorophores presumably represents either Zn released from proteins as the cell dismantles itself or Zn released from internal stores as organelles are degraded. Keeping this Zn sequestered away from

the Zn-sensitive caspases and other cell death executioner proteins is likely to be a critical and full-time job for Zn transporters. Is Zn transporter expression up-regulated early in apoptosis to achieve this goal? This should be easily addressed by real-time PCR studies, but since ribonucleases are also active during cell death, interpretations will need to be carefully made and supported by immunoblotting and immunocytochemistry.

In the light of evidence that Zn deficiency impairs the function of macrophages and presumably therefore impairs clearance of apoptotic cells, we need to re-examine some of the conclusions made from *in vivo* Zn deficiency experiments on apoptosis. For example, to what extent was impaired clearance of apoptotic cells, as opposed to heightened increase in the induction of apoptosis, a factor in the greatly increased frequency of apoptotic cells in the airway epithelium of asthmatic mice with restricted dietary intake of Zn [164]?

One of the gaps in our understanding of Zn-regulated apoptosis and in interpretation of many of the published experiments is the lack of data concerning the actual levels of the intracellular Zn pools. Few studies have attempted to measure intracellular Zn pools when assessing effects of Zn chelators, Zn-free medium or Zn supplements *in vitro* on apoptosis. It is not known whether the pools of Zn responsible for suppression of apoptosis are compartmentalized and, if so, what the local available concentrations of Zn are. It is also not known whether Zn supplementation affects the same pools and molecular targets within the apoptosis pathway as does Zn depletion. Compounding the problem is the uncertainty of the relevant pools to measure. Should we be measuring cytosolic free Zn, mitochondrial Zn, vesicular Zn, nuclear Zn or other Zn pools? How will these pools be measured? The recent demonstration [173] that in pancreatic islet cells, fluo-zin-3 measures the cytosolic pools while Zinquin measures the granular pools is a step in the right direction and offers hope that one day we will have a panel of Zn fluoropores available that measure different pools of intracellular labile Zn.

The dual roles of Zn in regulating cell proliferation and cell death point to a pivotal role for this metal ion in tissue homeostasis with important implications for diseases in which the delicate balance between cell birth and cell death goes amiss. Elucidating the mechanisms by which Zn and Zn transporters control these fundamental cellular processes presents a major challenge for Zn researchers over the next few years.

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6. Zinc Signaling

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Abstract. The majority of cellular zinc is tightly bound to proteins, where it has either catalytic or structural functions. Nevertheless, a regulatory role for zinc also exists, which is mediated by the small fraction of free or loosely bound zinc. This chapter discusses the role of free zinc in signal transduction. Different types of zinc signals were observed in eukaryotic cells, ranging from fast changes in less than a minute to signals that accompany cellular differentiation and last for several days. A range of molecular targets for these zinc signals has been identified, including a regulation of the activity of several kinases, phosphatases, phosphodiesterases, caspases, and transcription factors.

Keywords. Kinase; Protein tyrosine phosphatase; Second messenger; Signal transduction

Introduction

The zinc content varies considerably between tissues, from 10 µg/g dry weight in the brain to 100 µg/g in bone [1]. Plasma zinc concentrations are only around 1 µg/g, making it a predominantly intracellular ion [2]. In cultured cells, zinc is estimated to have an average concentration of several hundred micromolar; from 180 µM in the cell line BHK [3] to over 600 µM in rodent tumor cells [4], thereby significantly exceeding the zinc content of the surrounding culture media. The vast majority of cellular zinc is bound to proteins. So far, more than 300 enzymes and an even higher number of other proteins were found to contain zinc. Based on a search for known zinc binding sequences, it has been estimated that up to 10% of the proteins encoded in the human genome may contain zinc [5].

Tightly protein-bound zinc is required for catalytic, co-catalytic, and structural functions of enzymes [6]. Moreover, stabilization of structural domains, such as in zinc fingers and related structures, enables interaction of proteins with nucleic acids, as for many transcription factors, or an interaction between proteins [7]. The number of zinc proteins is as remarkable as the diversity of their respective functions, leaving no doubt that every aspect of life involves zinc. Accordingly, zinc is essential for humans [8,9], and zinc deficiency is a major cause for morbidity and mortality [10].

The essentiality of zinc seems not to be based exclusively on the protein bound fraction, but also on signaling by free zinc ions. Even though the notion of zinc being a part of signal transduction has gained momentum only in recent years, a respective function had already been suggested more than 30 years ago. Data indicated that zinc

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could be a second messenger [11], and, only a few years later, protein kinase C was identified as a zinc-regulated signaling enzyme [12].

A transmission of information by zinc could occur during paracrine communication between neighboring cells, or as a hormone-like function on the systemic level, but also as signal transduction within a cell. So far, paracrine functions were described in the central nervous system and pancreas. In the brain, zinc is released together with glutamate from a subset of glutamatergic neurons and may act as a neuromodulator [13-15]. In the pancreas, glucagon secretion by alpha cells is regulated by the zinc/insulin complex secreted from neighboring β -cells, and it was recently reported that this regulation is not due to insulin, but zinc [16,17]. Even though zinc has insulinomimetic effects, a hormone-like function has not been demonstrated, and there exists no indication that there may be one [2]. In contrast to limited involvement of zinc in intercellular communication, evidence has been accumulating that zinc is a vital component of intracellular signal transduction. This chapter aims to provide a brief overview of zinc's role in signaling within cells, describing zinc signals and their molecular targets.

1. Measurement of Free Zinc

Zinc signals can be defined as a change in the concentration of intracellular free zinc, affecting the transduction or processing of information on the cellular level. Most of these signals occur in the cytosol or nucleus. They can result from the transport of zinc through the plasma membrane, exchange with intracellular organelles or zinc binding proteins, or a combination of these mechanisms.

The investigation of zinc signals was greatly facilitated by the development of fluorescent sensors that detect changes in the availability of free zinc by a change of their fluorescent properties. The following paragraph briefly discusses some relevant discoveries and principles, a detailed overview can be found in chapter 9.

1.1. Fluorescent Zinc Probes

Early indication that fluorescent probes could detect significant levels of free intracellular zinc came from the investigation of calcium signaling. A well-known but frequently disregarded fact is that most probes that were developed and used for measuring calcium signals do also bind zinc. Zinc binds with a 3000-fold higher affinity than calcium to quin-2, thereby quenching its fluorescence. In two rodent tumor cell lines and primary leukocytes, the membrane-permeable zinc chelator TPEN [N,N,N',N' -tetrakis(2-pyridylmethyl)ethylenediamine] was used to remove zinc from quin-2, leading to increased fluorescence signals. These experiments demonstrated that free intracellular zinc had led to an underestimation of the calcium concentration [4]. FURA-2 has about 100-fold higher affinity for zinc than for calcium, and binding of both ions results in a fluorescent signal [18]. Hereby, zinc causes a shift in the excitation wavelength similar to calcium, making it virtually impossible to distinguish both ions during live-cell measurements [19]. Hence, zinc can contribute to signals believed to be caused by calcium [20,21], and in the case of thimerosal-induced signals measured by FURA-2 and Fluo-4, the fluorescence that had initially been thought to be caused by calcium, was in fact completely due to an intracellular release of zinc [22]. Notably, most fluorescent probes used for the detection of zinc are unaffected by

calcium [22-24], making it highly unlikely that a calcium signal could be mistaken for zinc.

The first specific zinc-probes were TSQ [25] and the chemically related Zinquin [23]. Fluorescence microscopy with these probes demonstrated that free zinc is not evenly distributed within cells, but there is considerable intracellular sequestration, frequently leading to lower nuclear zinc, and accumulation in vesicles, so-called ‘zincosomes’. This has since been confirmed with other probes, including FluoZin-3 and the ZinPyr family, which have been used to measure zinc signals in a variety of different cell types. In addition, recent developments such as ratiometric and/or protein-based genetically encoded sensors will allow an even more sophisticated analysis in the future [26].

1.2. Free Zinc

‘Free zinc’ is an operative term, used to distinguish the zinc involved in signal transduction from the tightly protein bound zinc that is thermodynamically unavailable. The term is used in this chapter with the understanding that it is chemically incorrect, because zinc ions will never be completely without ligands within a cell, but form complexes with amino acids, glutathione, phosphate, or several other low molecular weight ligands, when it is not bound to proteins. The free zinc pool has been given several different names, such as ‘labile zinc’, ‘mobile zinc’, ‘available zinc’ or ‘loosely bound zinc’.

Remarkably, it has been difficult to even determine the concentration of free intracellular zinc with certainty. Experiments by the group of O’Halloran with the zinc-binding regulator proteins Zur and ZntR from *Escherichia coli* have indicated femtomolar concentrations of free zinc [27]. This poses a major problem. Due to the small cellular volume, such low concentrations would result in less than one free zinc ion per cell, which is certainly not sufficient to interact with a number of regulatory binding sites to transmit information.

Notably, these values were determined for proteins from *E. coli*, and while some facts indicate that prokaryotes may use calcium as a signal [28], this is not necessarily the case for zinc. In contrast to Zur and ZntR from *E. coli*, most stability constants for eukaryotic proteins are not femtomolar, but lie between pK_D 10 and pK_D 12 [29]. Thermodynamically, femtomolar free zinc would mean that all these proteins would exist in their apo-form. This contradicts the fact that numerous metalloenzymes have been isolated from biological material in their zinc-bound form [6].

Measurements of free zinc concentrations with fluorescent probes in eukaryotic cells indicate that these concentrations lie several orders of magnitude above the femtomolar range. In 1985, Roger Tsien and his colleagues published a paper introducing a group of new fluorescent probes for calcium that included FURA-2 [18]. Herein, they also introduced a method allowing calculating free ion concentrations based on fluorescence measurements, given that a number of experimental conditions are met. This formula (1)

$$[Zn] = K_D \times \frac{F - F_{\min}}{F_{\max} - F} \quad (1)$$

can also be applied to zinc probes, such as FluoZin-3. Today, it is widely accepted that free zinc in most eukaryotic cells lies in the high picomolar to low nanomolar concentration range; examples are given in table 1.

Table 1. Free resting zinc levels in eukaryotic cells determined with fluorescent probes.

Cell type	Probe	Free zinc	Reference
<i>Primary cells</i>			
Muscle	FURA-2	0.1 nM	[30]
Neurons	MagFura-5	~2 nM	[31]
Cardiomyocytes	FURA-2	0.52 nM	[32]
Monocytes	FluoZin-3	0.17 nM	[33]
Lymphocytes	FluoZin-3	0.35 nM	[33]
<i>Cell cultures</i>			
HT-29	FluoZin-3	0.78 nM	[34]
PC-12	FluoZin-3	0.79 nM	[35]
Jurkat	FluoZin-3	0.14 nM	[33]
Raw 264.7	FluoZin-3	1 nM	[36]
HEP-2	FluoZin-3	0.5 nM	[37]

2. Investigation of Zinc Signals

As a signal, the level of free zinc needs to be tightly controlled. To this end, a number of proteins exist, solely to maintain cellular zinc homeostasis. This includes transporter proteins from the ZnT and Zip families, which are discussed in detail in chapter 8, but also zinc-binding proteins, such as the metallothioneins or calprotectin. Together, these proteins form a complex homeostatic system. Recently, this system was described in modeling studies, which are a promising tool for a better understanding of zinc signals [38]. So far, three major sources for zinc signals have been identified, i.e. uptake from the extracellular environment, reversible storage in vesicles, and oxidative release from storage proteins (figure 1). These will now be discussed in the context of their regulation and triggering agents.

2.1. Zinc Uptake

Early reports identified zinc uptake as an important regulatory event, which could be investigated by removal of zinc from the extracellular medium, even before specific fluorescent probes for zinc were available. It was demonstrated that zinc influx is required as a signal during proliferation [11] and cell differentiation [39]. In addition, zinc may also be taken up during neurotransmission, as described in chapter 10.

Despite obvious parallels to calcium signaling, there is one profound difference. Because the millimolar extracellular calcium concentration exceeds the nanomolar intracellular levels by far, passive influx can generate cytoplasmic calcium signals [28]. Extracellular zinc, however, is approximately one order of magnitude lower than the cellular concentration. The majority of both intra- and extracellular zinc is bound to proteins, leading to nanomolar free levels on both sides of the plasma membrane. Hence, in contrast to calcium, the uptake of zinc for signaling purposes should involve an active transport mechanism.

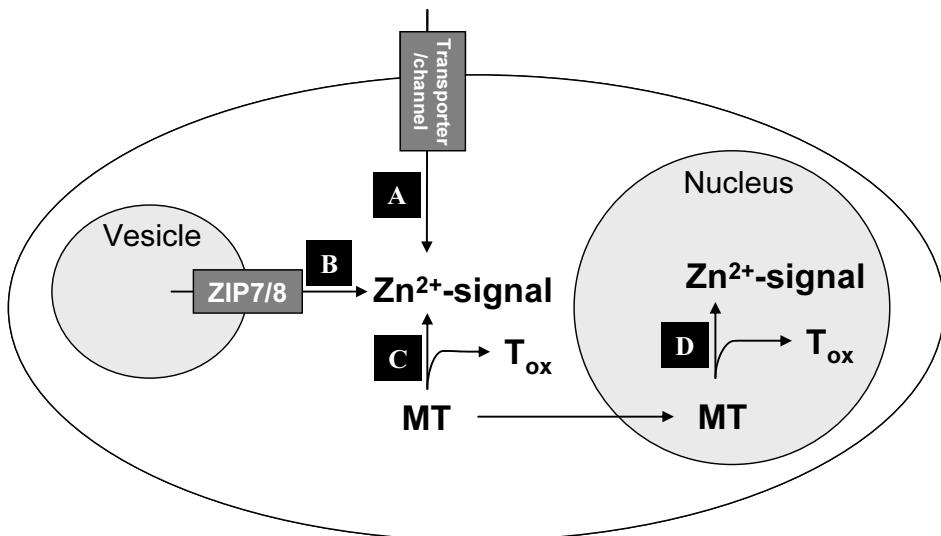


Figure 1. Sources of zinc signals. Zinc signals originate from different pools of zinc. (A) Influx of extracellular zinc mediated by transporters or ion channels. (B) Release of vesicular zinc. (C and D) Protein-bound zinc is released by oxidation of metallothionein (MT) into its oxidized forms, including Thionin (T_{ox}). This may occur either in the cytoplasm (C) or as a localized signal in the nucleus (D).

2.2. Vesicular Zinc

Fluorescent probes indicate accumulation of high amounts of free zinc in vesicular stores [23]. Based on the acidic nature of these zinkosomes, which were also positive for the vesicular transporter ZnT-2, it has been suggested that there is active, transporter-mediated accumulation of zinc in lysosomes [40]. Vesicular storage is reversible, because zinc can be released from zinkosomes upon stimulation with growth factors [41] and may also be released from the endoplasmic reticulum during the ‘zinc wave’ in mast cells [42]. Furthermore, FluoZin-3 labels zinkosomes in T-cells, which co-localize with staining for lysosomes. Zinc is released from these vesicles into the cytosol after stimulation of the T-cell-receptor or the interleukin (IL)-2-receptor [43,44].

Recently, intact zinkosomes were isolated and investigated by analysis of their X-ray absorption fine structure (XAFS), indicating that vesicular zinc is not free, but bound to an environment of sulfur, oxygen, and nitrogen ligands [45]. This binding environment most likely forms a ‘zinc sink’, which facilitates the accumulation of zinc against a considerable concentration gradient and reduces the amount of energy required for its storage.

2.3. Zinc-Release from Proteins by Reactive Oxygen or Nitrogen Species

In biological systems, zinc is redox inactive and only exists as the Zn^{2+} ion. Zinc binding by proteins is mediated by four amino acid side chains, coordinating zinc with oxygen (Glu, Asp), nitrogen (His) or sulfur (Cys). The latter can be oxidized. Hence, whereas zinc itself is insensitive toward redox biochemistry, its thiol-containing

binding sites are not. Therefore, these binding sites can be considered ‘molecular redox switches’, which can release or bind zinc upon oxidation or reduction, respectively [46]. Metallothionein (MT) is a small, cysteine-rich protein of 6-7 kD that can bind up to seven zinc ions in a zinc-sulfur cluster structure. It contains a considerable fraction of cellular zinc, which it can buffer over a wide concentration range by a combination of binding sites with different pico- to nanomolar affinities [47]. Furthermore, oxidation and oxidative polymerization of the molecule with loss of its zinc binding capacity constitute a link between zinc and redox signals [48,49].

Several reports have shown that oxidation leads to an intracellular zinc release, either by treatment with hypochlorous acid, hydrogen peroxide or aldehydes [50–52]. Another important biological oxidant is nitric monoxide (NO). It is best known for its function as a vasodilator, resulting from its interaction with the soluble guanylate cyclase heme group [53]. In accordance with this function, it has been suggested that NO might lead to zinc release through activation of the cGMP/PKG pathway, opening mitochondrial potassium ATP channels [54]. In contrast, most studies describe a release of protein-bound zinc from MT or zinc fingers by oxidation of cysteine thiols [55], and treatment of cells with nitric monoxide donors leads to a massive increase of free intracellular zinc [56,57]. Similar observations have also been made with peroxynitrite a highly reactive compound that is formed *in vivo* by a reaction of NO with superoxide [58].

In an elegant study, it was shown that endothelial cells treated with a combination of the pro-inflammatory cytokines tumor necrosis factor (TNF)- α , IL-1 β , and interferon (IFN)- γ , induce the expression of the inducible nitric oxide synthase (iNOS) and nuclear translocation of MT. iNOS-derived NO leads to a release of zinc from MT and a subsequent accumulation of nuclear free zinc [59].

2.4. Types of Different Zinc Signals

Calcium signals consist of a fast release from the ER or influx through the plasma membrane, occurring immediately after the cells were stimulated. Such signals are usually short-lived, lasting only up to a few seconds [28]. This is different for zinc signals. Not only are zinc signals generally slower than calcium, but, as depicted in figure 2, there is a range between fast zinc signals (occurring within less than a minute), an intermediate form (the ‘zinc wave’), to slow signals (lasting for days).

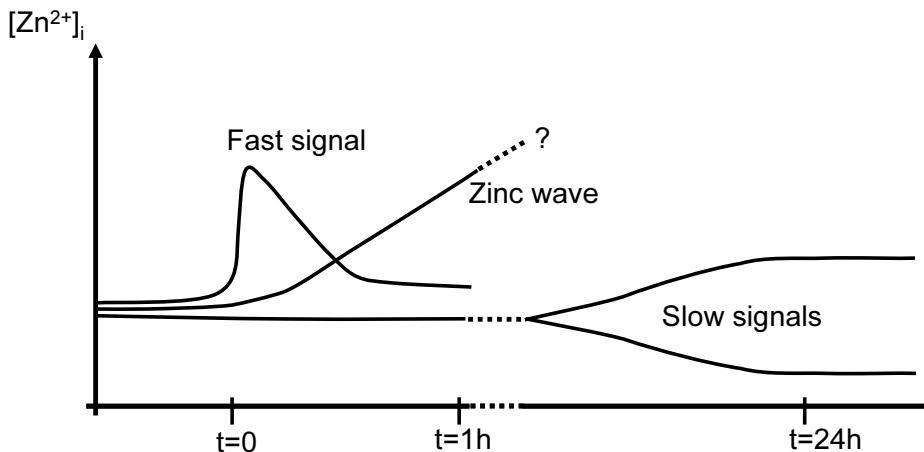


Figure 2. Different time courses of zinc signals. Schematic representation of different types of zinc signals. So far, three main types of zinc signals have been described. Fast signals start instantly after stimulation of cells, reaching their maxima within minutes. The zinc wave starts after a short lag phase and continually increases for at least one hour, with its further development being unknown. Finally, slow signals take more time to develop and can last for one or more days.

In some cases, it was possible to trigger the different types of zinc signals using ligands for cellular receptors, demonstrating that all three types can indeed occur in response to physiological stimulation (table 2).

Table 2. Examples for receptor-mediated zinc signals. ABs: Crosslinking antibodies

Stimulant	Cell type	Signal type	Reference
LPS	Monocytes	Fast signal	[36]
MCP-1	THP-1 cell line	Fast signal	[60]
IL-2	CTLL-2 cell line	Fast signal	[44]
ABs against FCε-R1	Mast cells	Zinc wave	[42]
ABs against T-cell-receptor	T-cells	Slow signal	[43]
NGF	PC12 cell line	Slow signal	[35]
LPS	Dendritic cells	Slow signal	[61]
IL-1β, TNF-α, and IFN-γ	Aortic endothelial cells	Slow signal (nuclear)	[59]
Calcitriol	HL-60 cell line	Slow signal	[62]

Fast zinc signals were observed after electrical stimulation by influx through voltage-dependent calcium-channels in cardiomyocytes [30]. Other reports describe the rapid release of zinc from intracellular stores, e.g. in NIH 3T3 cells stimulated with PMA [51], from monocytes and granulocytes exposed to bacterial structures that serve as a danger signal for infection [36], or in T-cells by stimulation of their receptor for the cytokine IL-2 [44].

The slower zinc wave develops after a few minutes lag time. It has been observed in mast cells after stimulation of the Fcε-receptor 1 with crosslinking antibodies [42]. Here, free intracellular zinc rises for at least one hour after the onset of its release from intracellular structures, most likely the endoplasmic reticulum.

Finally, slow signals usually depend on changes in the expression of proteins involved in zinc homeostasis, such as transporters, storage proteins or nitric oxide

synthase. These signals last for several hours, if not days after stimulation of cells, and are predominantly observed in cell proliferation or differentiation [11,35,39,61-64].

2.5. Role of Zinc Transporters in Zinc Signaling

The growing number of known zinc transport proteins has led to a closer investigation of their role in signaling. In *Caenorhabditis elegans*, it was shown that CDF-1, which acts similar to the mammalian transporter ZnT-1, stimulates Ras signaling by mediating zinc efflux, indicating a negative regulation of Ras-mediated signaling by zinc [65]. Later, it was found that zinc promotes the inhibitory phosphorylation of Ras on serine 259 [66]. In another study, ZnT-1 has been shown to form a complex with the transmembrane channel-like proteins EVER1 and EVER2, regulating nuclear zinc, the activity of zinc-dependent transcription factors, and proliferation of keratinocytes [67].

A function for Zip7 as a central regulator of zinc efflux from the endoplasmic reticulum into the cytoplasm has recently been demonstrated in breast cancer cells, indicating an important role for this transporter in the generation of zinc signals [68]. A similar role in the release of zinc from lysosomal compartments has been found for Zip 8 in T-cells. Here, Zip8 releases zinc into the cytoplasm, where it is required for the production of IFN- γ in response to stimulation of the T-cell-receptor [69].

Over expression studies of zinc transporters indicate their involvement in the regulation of several signaling processes, including the inhibition of NF- κ B by Zip1 and expression of cyclinD1, IL6, and STAT3 in response to over expression of Zip4 [70,71].

2.6. Tools for the Modulation of Zinc Signals

To investigate the biological role of zinc signals, it is not sufficient to be able to observe them, rather it is useful to actively sequester or induce them in order to elucidate their functions. Sequestration of zinc is efficiently achieved by chelating agents. Due to its membrane permeability and high affinity for zinc, TPEN is frequently the chelator of choice, and has been used to demonstrate the zinc-dependence of p53 folding, the interference of zinc with calcium measurements, and the requirement of zinc signals for mitogen activated protein kinase (MAPK) activation [4,36,72]. However, no chelator is absolutely specific and, in addition to its high affinity for zinc ($10^{15.58} \text{ M}^{-1}$), TPEN chelates other bivalent cations as well, including Mg^{2+} , Ca^{2+} , Fe^{2+} , and Cu^{2+} with respective affinities of $10^{1.7} \text{ M}^{-1}$, $10^{4.4} \text{ M}^{-1}$, $10^{14.61} \text{ M}^{-1}$, or $10^{20.54} \text{ M}^{-1}$ [4,73].

If an effect of TPEN is really based on its zinc chelating ability, addition of the zinc-saturated chelator should have no effect. In one instance it was described that the induction of apoptosis in the cell line PC12 was observed with TPEN as well as its zinc complex, and therefore independent from its zinc chelating ability. Here, TPEN could act either as a chelator for metal ions other than zinc, or completely independent from its metal binding capability [74]. Consequently, specificity is an important issue when chelators such as TPEN are applied.

An affinity exceeding that of most zinc binding proteins gives rise to the concern that the effects of TPEN might not be mediated by chelating zinc signals, but instead by removing tightly protein bound zinc that does not function as a signal. A recent study demonstrated the removal of zinc from zinc finger proteins by TPEN *in vitro*. It

found reduced DNA-binding capacity of the zinc finger transcription factor Zn₃-SP1 isolated from TPEN-treated LLCPK1 cells [75]. However, this does not mean that the non-toxic low micromolar concentrations of TPEN used in most studies lead to massive depletion of zinc from metalloproteins. Incubation of LLCPK1 cells with up to 100 µM TPEN for 30 minutes had no effect on DNA-binding of Zn₃-SP1. Even after 24 hours, 30 µM TPEN were required to affect DNA binding [75]. The difference to the *in vitro* results can be explained by two reasons: (I) The total cellular zinc concentration is several hundred micromolar [3,4], and TPEN will preferentially chelate the fraction with the highest availability, i.e. free zinc. (II) Most cell culture media contain low micromolar concentrations of zinc, thereby saturating a fraction of the TPEN before it even reaches the cells. Hence, low micromolar concentrations of TPEN will not be sufficient to remove a large fraction of tightly protein bound zinc, such as the one in zinc fingers, but will predominantly interact with loosely bound zinc.

In addition to TPEN, a number of other membrane-permeable chelators has been used as well, e.g. 1,10 phenanthroline for investigating the role of zinc binding of the transcription factor p53 or the zinc-dependent interaction between the kinase Lck and CD44 [76]. Furthermore, 2,3-dimercapto-1-propanesulfonic acid was used to demonstrate that free zinc is required for chemokine production by the airway epithelium [77].

Zinc homeostasis controls the amount of zinc crossing the plasma membrane. Hence, for imitating zinc signals, an additional tool is required to overcome homeostatic control. Addition of zinc together with ionophores, such as pyrithione, shuttles zinc as a complex through the plasma membrane without participation of zinc transport proteins. In this respect, it should also be noted that pyrithione, applied in the absence of zinc, acts as a chelator and may cause the same biological effects as TPEN [62]. A similar duality also exists for Clioquinol, which is a promising lead structure in the therapy of Alzheimer's disease, where it has been suggested to act either as a ionophore or chelator [78].

3. Molecular Targets of Zinc Signaling

Imitating zinc signals by addition of zinc to cells, frequently in the presence of an ionophore, has been reported to activate a myriad of signaling proteins. Due to the cascade-like nature of signaling pathways, any activation of a single signaling protein at the level of a receptor (or at least close to it), will lead to the subsequent activation of numerous downstream signaling pathways. A comprehensive list of all signaling proteins indirectly affected by zinc would exceed the available space. Instead, the following section aims to introduce a number of important molecular targets, highlighting different principles by which zinc interacts with signal transduction (figure 3).

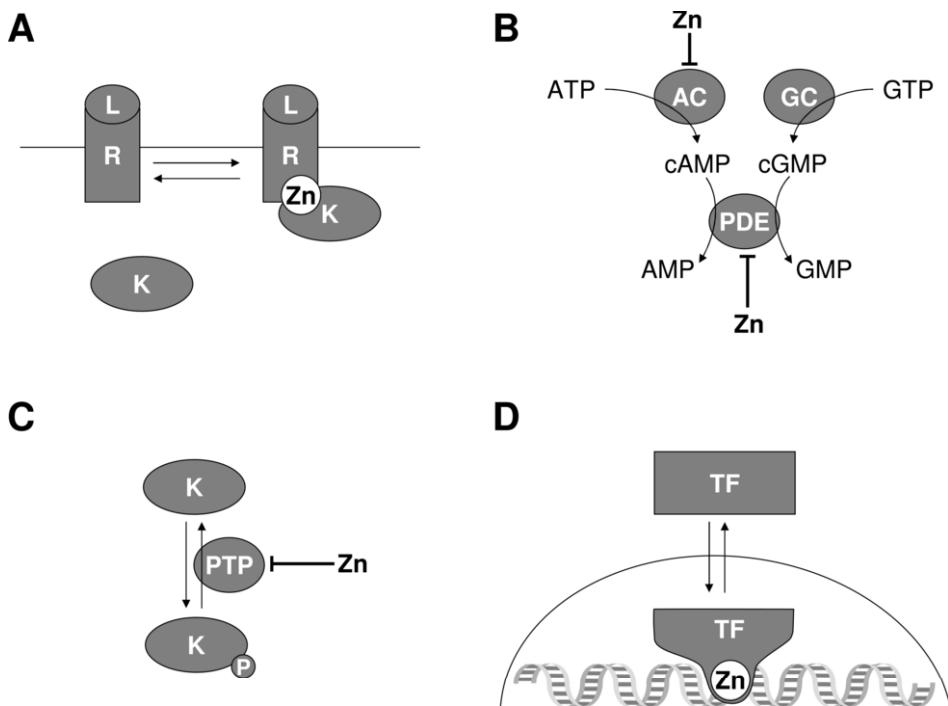


Figure 3. Interaction of zinc with signaling pathways. Zinc signals interact with signaling pathways using different principles. This includes the stabilization of multiprotein complexes (A), modulation of second messenger metabolism by inhibiting synthesis or degradation (B), preserving protein phosphorylation by inhibiting protein tyrosine phosphatases (C), or inducing functionally relevant conformational changes by binding to proteins. Abbreviations: AC, adenylate cyclase; GC: guanylate cyclase; K, kinase; L, ligand; P, phosphate group; PDE, cyclic nucleotide phosphodiesterase; PTP, protein tyrosine phosphatase; TF, transcription factor.

3.1. Receptors

Many molecules that transmit information between cells (e.g. growth factors) are sensed by receptors on the plasma membrane, which then initiate intracellular signaling cascades. Extracellular zinc has been shown to inhibit the release of the second messenger calcium triggered by several different receptors [79-81]. On the other hand, it has also been reported that extracellular zinc can trigger receptors [82-84]. These so-called ‘zinc-sensing receptors’ trigger a G_q protein- and phospholipase C-dependent release of calcium from thapsigargin-sensitive intracellular calcium stores, as well as MAPK and PI3K signaling [82,85]. Recently, it has been found that zinc triggers the G protein-coupled receptor 39 (GPR39) by binding to two histidine residues of its extracellular domain, suggesting that GPR39 might be the zinc sensing receptor [86,87].

3.2. Second Messengers

Second messengers are diffusible chemical signaling molecules. The first one to be discovered was 3',5'-cyclic adenosine monophosphate (cAMP). It is formed from ATP by adenylate cyclases (AC), and mediates its signaling function by binding to the

regulatory subunits of protein kinase A (PKA), leading to their dissociation and subsequent activation of the kinase. To terminate the signal, cAMP is degraded by cyclic nucleotide phosphodiesterases (PDE), which hydrolyze the ester bond in the 3' position, resulting in the formation of AMP. Both AC and PDE are regulated by zinc.

The catalytic activity of AC is inhibited by zinc. Whereas this has been confirmed in several independent observations, the mechanism still remains to be identified [62,88,89]. One report describes binding of zinc to a site on the AC, thereby causing direct, reversible inhibition of the enzyme [90]. In contrast, another study identified the generation of a nucleotide-free form of the stimulatory GTP-binding protein of AC, which is inactive with regard to AC activation, as the molecular basis for the inhibition of AC signaling by zinc [91].

The activity of isolated PDEs is augmented as well as reduced by addition of zinc, depending on its concentration. This indicates a requirement of zinc for enzymatic activity, but also inhibitory effects [92,93]. Addition of zinc to cellular lysate inhibits cyclic nucleotide degradation, suggesting that a rise of cellular zinc will most likely block PDE activity [89]. Notably, just as cAMP, cGMP is also cleaved by PDEs and zinc inhibits the degradation of both second messengers. Regardless, zinc treatment of cells can lead to reduced levels of cAMP, indicating that inhibition of its synthesis overrules the inhibition of its degradation, whereas levels of cGMP are elevated due to inhibition of its degradation alone [66,89].

Another second messenger is calcium. Zinc inhibits the uptake of calcium by isolated hepatic nuclei, potentially by interacting with thiol groups in nucleic calcium transport proteins [94]. In contrast to zinc, which acts directly on its target proteins, most effects of calcium are mediated by Calmodulin (CaM). Binding of calcium to its four binding sites on CaM leads to a conformational change of the protein, which then binds to its targets, including calcium/CaM-dependent protein kinase (CaMPK), the phosphatase calcineurin, or adenylyl cyclase. CaM has six additional binding sites for bivalent metal ions, and zinc binds to CaM either via these or, because of its similar ionic radius, via the calcium binding sites [95-98]. Zinc can bind to CaM and alter its structure, potentiating the biological activity of the $\text{Ca}^{2+}/\text{CaM}$ complex [97]. The activation of CaM-dependent phosphodiesterase activity by zinc involves CaM and the zinc-mediated activation was blocked by the calmodulin-inhibitor trifluoperazine, demonstrating that $\text{Zn}^{2+}/\text{CaM}$ activates PDE1 [96]. On the other hand, zinc has been shown to inhibit CaMPK-II by a mechanism involving autophosphorylation of Ser279 [99,100]. Moreover, the same reports that found inhibitory effects, also reported activation by zinc when different concentrations were used. Notably, these effects seem to be independent from an interaction with CaM [99,100].

3.3. Kinases

A simplified view of phosphorylation signaling involves cascades of kinases, which are activated upon phosphorylation and then phosphorylate subsequent kinases in that pathway. Many kinases were shown to be activated after cells had been treated with zinc, but in most instances zinc interacted with some signaling molecule upstream of the one investigated, instead of activating it directly. A number of kinases which are direct molecular targets are discussed below.

A role for zinc in signal transduction has been suggested based on its requirement for protein kinase C (PKC) activation [101]. In several instances it was shown that zinc-chelators, such as TPEN, inhibited the activation of PKC [102-104], and that

PKC-activity is reduced in zinc-deficient cells [105]. On the other hand, addition of zinc stimulates PKC translocation to the cytoskeleton, an important step during PKC activation [106-108].

In the N-terminal regulatory PKC-domain, four zinc ions are bound by the kinase, each by one nitrogen (histidine) and three sulfur (cysteine) ligands [109,110]. Structural stabilization by these four zinc ions may form the basis for PKC-regulation by zinc. Zinc augments the binding of phorbol esters to the regulatory, zinc-containing domain of PKC [111,112], and mutational analysis of the zinc binding amino acids demonstrated their requirement for the binding of phorbol esters [113,114]. Moreover, oxidative zinc-release from the zinc-containing regulatory domain of PKC led to a loss of sensitivity toward its cofactors [115]. However, these zinc ions are relatively tightly bound [109], suggesting the existence of either auxiliary mechanisms for regulating the zinc content of PKC, or of additional regulatory zinc binding sites.

Not only is PKC activity regulated by zinc, this kinase can also be the source of zinc signals. Zinc can be released directly from the regulatory domain of PKC [116,117], or be redistributed in the cell upon activation of PKC. Phorbol esters triggered an increase of free zinc [33,51,118], redistribution of zinc from the nucleus and mitochondria to the cytosol and microsomes [119], and a transient increase of nuclear zinc, caused by PKC β [120].

A different molecular mechanism for regulation by zinc has been described for the Src-family kinase Lck. It is essential for proximal T-cell receptor (TCR) signal transduction during development and activation of T-lymphocytes. Src-family kinases consist of an N-terminal site for palmytoylation or myristoylation, a unique region, the regulatory Src-homology (SH) domains SH3 and SH2, the tyrosine kinase domain SH1, and a negative regulatory domain at the C-terminus [121].

Lck differs from all other Src kinases by being regulated through two zinc-dependent protein interface sites. Upon recruitment to the TCR signaling complex, Lcks transphosphorylate their activation loop tyrosine 394 of the SH1 domain, promoting an active conformation, which leads to a 2-4-fold increase of catalytic activity. This phosphorylation of Lck is augmented by zinc ions, even in the absence of any additional proteins [122]. Structural analysis revealed that zinc ions bind histidine and aspartate residues from SH3 domains of two Lck molecules, inducing their homodimerization. This most likely promotes transphosphorylation of tyrosine 394 by enhancing Lck clustering. Notably, the dissociation constant of zinc from the Lck dimer was found to be 100 nM or less, indicating that it could occur at physiological zinc concentrations [123].

The unique N-terminal region of Lck contains a dicysteine motif that mediates its binding to the coreceptors CD4 and CD8, thereby recruiting Lck to its target proteins in the TCR signaling complex. This interaction was disrupted by the chelator o-phenanthroline, indicating that the cysteine residues might cause interprotein association by the coordination of metal ions such as zinc [124]. Later, structural analysis of complexes between Lck, zinc, and CD4/CD8 showed that zinc is coordinated in a ‘zinc clasp’ structure by four cysteines: two thiols are provided by a CxCP motif in CD4 or CD8, whereas the others are from a CxxC motif in the unique N-terminal region of Lck [125]. Recently, it has been reported that Lck is also recruited to the cytoplasmic domain of the adhesion molecule CD44 in a zinc-dependent manner [126]. In contrast to CD4/CD8, no cysteine residues from CD44 were essential for this association.

Among the Src-family kinases, zinc-regulation is specific for Lck. The zinc binding residues in the unique and SH3 domains are only found in Lck [121,123]. Furthermore, only the association of CD44 with Lck, but not with the Src-family kinase Fyn, is inhibited by the chelator o-phenanthroline [126].

3.4. Phosphatases

Phosphorylation signals can be caused by augmented kinase activity, but an equally important mechanism is signal termination by protein phosphatases. These enzymes are subject to regulation by gene expression, protein degradation, intracellular localization, ligand binding, and various posttranslational modifications [127]. One major group are the protein tyrosine phosphatases (PTP); 107 PTPs are encoded in the human genome [127]. A common feature is the signature motif (H)CX₅R. The cysteine is essential for catalysis, because cleavage of the phosphoester bond results from a nucleophilic attack by the cysteine thiolate on the phosphor atom. Based on the protein's microenvironment, the pKa of this cysteine is unusually low. This is necessary for catalytic activity, but also increases the susceptibility toward oxidation and should augment binding of metals such as zinc [128]. PTPs are well known to be inhibited by zinc [129]. Experiments with truncated SHP-1 confirmed that inhibition by zinc is mediated by an interaction with the catalytic domain and it has been suggested that zinc inhibits PTPs by binding to the active site thiolate [130].

The activity of several enzymes involved in signal transduction is affected by zinc. However, micro- or millimolar inhibition constants *in vitro* can not be seen as an indicator for a regulation by free zinc *in vivo*, where only nanomolar concentrations are found. In this respect, PTPs are a likely target for zinc signals, because inhibition constants for several different phosphatases were determined and found to be in the nanomolar concentration range [41]. Consequently, basal free intracellular zinc could partially inhibit PTP activity, and zinc signals would alter the ratio of active to zinc-inhibited phosphatases. This is supported by the observation that chelation of cellular zinc with TPEN abrogates phosphorylation of the insulin receptor and its downstream pathways, indicating a physiological role of free zinc in insulin signaling [130]. An involvement of phosphatase inhibition by zinc has also been demonstrated in epidermal growth factor receptor and Src signaling in airway epithelial cells, where zinc inhibits tyrosine phosphatases [131,132]. Furthermore, Zip7-mediated zinc release from intracellular storage sites has been proposed to influence tyrosine kinase signaling via inhibition of phosphatases [133]. One subgroup of the PTP superfamily are MAPK phosphatases (MKP). Several reports describe MKP inhibition as the molecular basis for the activation of MAPKs by zinc [36,44,134,135].

PTPs are the major group of zinc-regulated phosphatases, but reports indicate that other types may be affected as well. Calcineurin (CN) is not a PTP, but a serine phosphatase. Nevertheless, it is inhibited by zinc and has recently been shown to be sensitive to over-expression of the zinc transporter Zip8 [69].

3.5. Caspases

Caspases (cysteine-aspartic acid proteases) are central effector enzymes in apoptosis, which involves a cascade of proteolytic cleavage of inactive pro-caspases into the functional enzymes.

Following the initial report that caspase-3 is inhibited by zinc [136], it was shown that several caspases are inhibited by low micromolar zinc concentrations [137]. Among caspases -3, -6, -7, and -8, caspase-6 had the highest sensitivity, showing an apparent binding constant of 0.3 μ M [137]. Notably, this still exceeds physiological levels of free intracellular zinc, but additives to the reaction buffer, such as the thiol-based reducing agent β -mercaptoethanol, could have bound zinc and led to an overestimation of the metal ion concentration required for inhibition [137]. Measurements under optimized conditions found that the IC₅₀ of zinc for caspase-3 lies below 10 nM [138].

Control of caspase activity through inhibition by zinc is probably one mechanism of this metal's anti-apoptotic actions. These will not be discussed in detail here; instead the reader is referred to chapter 5 for an overview on the role of zinc in apoptosis. It should, however, be noted that in addition to their function in apoptosis, caspases are also involved in other signaling events, e.g. caspase 2 in cell cycle regulation, DNA repair, and cellular survival [139], or the so-called 'inflammatory caspases', such as caspase-1, which are involved in the processing of the cytokines IL-1 β and IL-18 by the NALP-3 inflammasome [140]. The potential impact zinc may have via caspases on functions other than apoptosis remains to be explored.

Despite their differences in function, caspases share one important feature with PTPs: an active site cysteine. Similar to the proposed mechanism of PTP-inhibition, it has been suggested that caspases are inhibited by zinc through binding to the active site cysteine-thiol [141].

3.6. Transcription Factors

Given the high prevalence of zinc-containing structural domains in transcription factors, the evidence for a direct impact of zinc signals on transcription factor activity is surprisingly limited. As stated above, even treatment with a high affinity chelator such as TPEN does not easily deplete zinc from zinc fingers, indicating that there is not much dynamic regulation based on the interaction of free zinc with this structural motif.

One exception is the metal response element-binding transcription factor (MTF)-1. MTF-1 has a total of six Cys₂His₂ zinc finger domains, two of which are not constitutively binding zinc. Hence, MTF-1 functions as a zinc sensor, because elevated availability of zinc leads to binding and stabilization of the two zinc fingers, resulting in the reversible induction of DNA-binding activity [142]. The DNA sequence recognized by MTF-1 is the metal response element (MRE) core consensus sequence TGC(G/A)CNC [143]. Among the more than 40 genes containing MREs in their promoter sequence, several have a role in zinc homeostasis; MTF-1 promotes ZnT-1 and MT transcription and may negatively regulate Zip-4 and -10 [144-147]. In addition to stabilization of its zinc fingers, MTF-1 is also indirectly regulated by zinc. Several kinases phosphorylate the transcription factor under conditions of elevated zinc availability, which is important for nuclear translocation of MTF-1 and its transcriptional activity [148,149].

The tumor suppressor p53 is another transcription factor that can be regulated by the availability of free zinc; it has been suggested that changes in the intracellular concentration of zinc regulate p53 activity [150]. p53 contains several cysteine residues, three of which are involved in zinc-binding [151], and zinc is required for p53 folding into its 'wild-type', i.e. DNA-binding, conformation [150]. Treatment of cultured cells with TPEN or o-phenanthroline resulted in the accumulation of a misfolded form of

p53 with reduced DNA binding, an effect that was reversible after removal of the chelator and addition of extracellular zinc [72,76,152].

At room temperature, zinc is not easily released from the DNA-binding domain of p53. However, it is rapidly lost at 37°C, potentially enabling exchange between p53 and other metal binding proteins, furthermore indicating that a considerable amount of cellular p53 may exist in the zinc-free state under conditions of reduced zinc availability [153].

One mechanism to control p53 activity could be regulation of its zinc content by MT. Elevated expression of MT deactivated p53, presumably by chelating zinc. In contrast, moderate over expression of MT activated p53 DNA-binding, suggesting a role for MT as chaperone that supplies zinc to p53 [152]. These differential effects of MT correspond well to the binding sites with different affinities [47]. Low affinity sites may supply zinc to p53, leading to its activation. In contrast, a drastic increase in the expression of MT leads to a number of new high affinity sites that remove zinc from proteins such as p53. Regulation of p53 activity by the protein HIPK2 also involves a zinc- and MT-dependent mechanism. HIPK2 is bound to the MT2A promoter as a co-repressor. Its downregulation leads to increased expression of MT2A, with subsequent inhibition of p53. In this experimental system, p53 wild-type conformation and transcription activity were restored by addition of zinc to the cell culture medium [154].

The transcription factor nuclear factor kappa B (NF-κB) is also a well known target of zinc in signaling. In its inactive state, NF-κB is a complex of three proteins. Two are members of the Rel/NF-κB family, which form hetero- or homodimers, constituting the actual transcription factor. The third is an inhibitor of kappa B (IkB) protein, which keeps NF-κB in an inactive state in the cytosol. Upon phosphorylation of serine residues of IkB by IkB kinases (IKK), IkB is ubiquitinylated and degraded by the proteasome. The remaining dimer of Rel/NF-κB proteins is then free to enter the nucleus, initiating NF-κB-dependent transcription.

Many studies have investigated the impact of zinc on NF-κB, with contradictory results. Activation of NF-κB by zinc was observed in epithelial cells [155], T-cells [156], microglia [157], and by the zinc signal in response to Fcε-1R or TLR-4 on mast cells or monocytes, respectively [36,158]. In contrast, zinc attenuated TNF-induced activation of NF-κB in mast cells [159] as well as in porcine endothelial cells [160]. It also inhibited the activation of NF-κB in response to LPS-stimulation in Kupffer cells [161] and monocytes/macrophages [66,162], and overexpression of the zinc import protein Zip1 inhibited NF-κB signaling in prostate cancer cells [70]. In addition, another report states that while zinc activates IkB phosphorylation and DNA binding activity of NF-κB, it also blocks its nuclear translocation, leading to an accumulation of the active transcription factor in the cytosol [163]. MT also affects NF-κB signaling. MT-knockout fibroblasts had less total cellular NF-κB, but higher levels of active NF-κB, compared to the respective wild type cells [164].

The activating effect of zinc has been explained by a function of this ion in DNA-binding of NF-κB [165]. However, NF-κB does not contain a zinc finger or related structural motif, and the crystal structures of the p50 homodimer and the p50/p65 heterodimer complexes with DNA do not indicate a role for zinc [166,167]. Some studies reported that metal ion chelators inhibit NF-κB DNA-binding *in vitro* [165,168]. However, several chelators with high affinity for zinc failed to impair DNA-binding of NF-κB [168,169]. Moreover, many buffers in which experiments with NF-κB are performed *in vitro* contain millimolar amounts of EDTA, which does not seem to affect the DNA-binding activity of the transcription factor.

Activation of upstream signaling pathways of NF- κ B has been observed in studies using HUT-78 cells, indicating that zinc affects I κ B phosphorylation rather than directly activating NF- κ B [156,170]. Another report indicated that zinc affects the phosphorylation of several serine residues on NF- κ B [155]. We found that a concentration of TPEN that did inhibit NF- κ B activation in LPS-treated monocytes also abrogated I κ B phosphorylation, but had no effect on DNA-binding after NF- κ B had been isolated from these cells [36]. Taken together, these results suggest that the requirement for zinc originates in regulating the signaling pathways leading to the activation of NF- κ B, rather than the activity of the transcription factor itself.

Several mechanisms have been proposed for the inhibitory effect of zinc on NF- κ B. They include direct inhibition of NF- κ B DNA binding, which was observed after addition of several hundred micromolar of zinc *in vitro*. These concentrations equal the total cellular zinc content and free zinc in this concentration range does not occur *in vivo* [168,171]. Lower concentrations of only a few micromolar inhibited IKK activity *in vitro* [162]. *In vivo*, zinc treatment reduced the LPS-induced phosphorylation of I κ B and NF- κ B, indicating that zinc acts further upstream in the signaling pathway [161]. In human monocytes, zinc abrogated LPS-induced NF- κ B activation by a mechanism depending on cyclic nucleotide-mediated activation of PKA, which subsequently inhibits Raf kinase and thereby the pathway for NF- κ B activation [66,89]. An alternative mechanism involves augmentation of mRNA and DNA-specific binding of the transactivating factor A20 by zinc, which acts as an inhibitor of NF- κ B activation [172,173].

Zinc has been shown to either activate or inhibit NF- κ B and this seems to depend, at least in part, on the concentration of zinc [174]. NF- κ B is most likely not a direct target for zinc, but under the control of several zinc-regulated pathways. The net effect of zinc on NF- κ B activity results from interaction of these upstream signal transduction pathways.

4. Conclusion and Perspectives

Different types of zinc signals have been observed, and seem to regulate a variety of cellular functions. The molecular targets identified for zinc in signal transduction already comprise an impressive number of proteins; yet, a lot of effects indicate that there may be even more. Future research will have to address the question how a relatively unspecific change of intracellular free zinc can differentially regulate multiple signaling pathways. Certainly, not every cell expresses all zinc-regulated proteins, but still many are expressed in parallel and need to be triggered in the correct signaling context. As shown in figure 4, different enzyme families (and potentially even members of one family) might respond to different zinc concentrations. Caspase-3 has a relatively low binding constant *in vitro*, and zinc could still inhibit caspases, and thereby apoptosis, while PTPs at large remain active. For PDEs, current *in vitro* measurements indicate low micromolar inhibition constants, but these may be lower in intact cells. Such a succession of regulatory concentrations corresponds to observations during differentiation and activation of monocytes. Here, undifferentiated cells have high free zinc, which decreases during differentiation, a process depending on cyclic nucleotide signaling [62]. Activation of mature monocytes involves PTP-inhibition [36]. Notably, the susceptibility of differentiated monocytes to apoptotic death is higher, which, among other factors, may result from less caspase-inhibition by zinc.

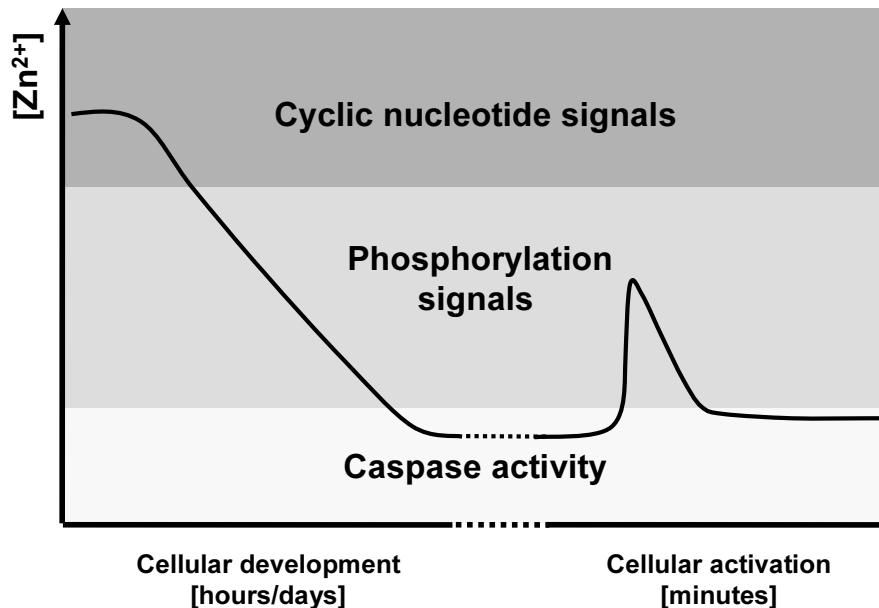


Figure 4. Model for the differential regulation of multiple signaling pathways by free zinc. Signaling enzymes may be regulated in different intracellular concentration ranges of free zinc, based on different affinities of their regulatory binding sites. One example is the differentiation of HL-60 cells from promyelocytes into monocytes, which is accompanied by a decrease in intracellular free zinc [62]. Undifferentiated cells have relatively high free zinc levels. Their decrease promotes differentiation based on the role of zinc in cAMP signaling [62]. Notably, differentiated cells show higher susceptibility toward apoptotic cell death [175], which might be based on a partial loss of caspase-inhibition by free zinc. Finally, activation of monocytes involves short term zinc signals which are required to protect phosphorylation signals by inhibiting PTPs [36].

Differential affinities will not be the only mechanism by which specificity is achieved. Another important aspect is sub-cellular localization. The existence of two dozen transport proteins, many of them on the membranes of organelles, indicates a tight control of sub-cellular distribution, and it has already been shown that zinc signals can be limited to a cellular compartment, such as the nucleus [59]. Still, many measurements of free zinc only give average values for the entire cell, or even total populations of cells, not reflecting spatial differences. Respective experiments accounting for such differences might add to our understanding on how specific regulation of zinc signaling takes place. Finally, it remains to be seen if zinc freely binds to all its regulatory sites, or if additional mechanisms such as chaperone proteins are involved, adding an additional layer of regulation and complexity.

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7. Zinc Modulation of Ion Channels

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Abstract. Divalent ions like zinc, in its chelatable form, are found ubiquitously in all tissues in the mammalian body. Its amount varies and in some tissues, such as certain areas of the brain and the gastrointestinal tract, it may reach hundreds of micromolar in a specific compartment. Physiological as well as pathological conditions can lead to large changes in both the extra- and intracellular concentration of this ion. Zinc modulates, enhances or inhibits many channels in the course of physiological activity as in the hippocampus or during pathological incidents such as during ischemic episodes. In this chapter we have tried to outline the effect on some of the channels modulated by zinc. Channel modulation is achieved by the slow permeation of zinc through the channel or, like in most cases, due to binding of zinc to specific sites on the channel proteins. We concentrated on major mono and divalent ligand or voltage gated cationic channels since space was not allowed to discuss all the channels, including anionic and non ionic channels modulated by zinc.

Keywords; voltage gated ion channels, ligand gated ion channels, TRP

Introduction

In living organisms lipid membranes serve as boundaries separating different hydrophilic milieu of the cells. For example, the plasma membrane separates the cell from the extracellular space, while the membranes of intracellular organelles, such as endoplasmic reticulum, lysosomes, mitochondria etc., separate them from the cytosol. Due to their lipophilic nature, biological membranes form barriers that prevent the free movement of ions and other hydrophilic molecules such as sugars and even water from one compartment to the other. Normal cellular functions critically depend on maintaining exact concentrations of these hydrophilic molecules in a given compartment, and therefore they are tightly regulated by a large number of proteins acting as pumps, transporters and channels. These proteins are expressed in all membranes regulating the transport of solutes into, and out of, the lipid enclaved hydrophilic compartments, thus allowing the proper execution of many biological processes. Among these processes are gene expression, signal transduction, cell excitation, hormone secretion and muscle contraction to name only a few. Zinc ions play an essential role in mammalian development and function. Indeed, zinc deficiency severely affects embryonic development and the proper function of multiple systems

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[1]. By interacting with specific zinc fingers, zinc ions play crucial roles in activating transcription factors [2, 3], and are essential for the activity of numerous enzymes, being present at the catalytic core [1]. In addition, zinc specifically binds and modulates the activity of membrane proteins, channels and transporters such as *N*-methyl *D*-aspartate (NMDA) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, the dopamine transporter, and many others [4]. But zinc also has a dark side. The massive influx of zinc, released during ischemic episodes from glutamatergic synaptic vesicles, in certain parts of the hippocampus and cortex, is considered a major factor in the ensuing of neuronal cell death [5-7]. Similarly, in seizures, and in traumatic brain injury massive zinc release occurs, and may also trigger neuronal death [8-11]. Zinc, therefore, appears to play an important role in multiple cellular functions by, among other proteins, modulating membrane channels either as an inhibitor or partial agonist or by changing the channel activity through binding to allosteric sites on the channel proteins. In this chapter we will outline some of the roles of zinc as modulator of a variety of ion channels. Due to space limitation we will deal only with the direct effect of zinc on some of the cationic channels. The indirect effect of zinc on signal transduction which also regulates ion channels is discussed in chapter 6. We shall not discuss the effect of zinc on anion channels such as glycine and gamma-aminobutyric acid (GABA_A) or its effect on non ionic water channels such as aquaporins, all of which are modulated by zinc.

1. Monovalent Channels

Monovalent ions such as sodium, potassium and hydrogen play a crucial role in vital biological process such as nerve and muscle excitation, hormone and fluid secretion. This is in addition to their major contribution to the osmotic power of physiological solutions as well as providing the correct environment for proper activity of the cellular machinery. Among the monovalent channels regulated by zinc are sodium, potassium and hydrogen ion channels.

1.1 Sodium Channels

Sodium channels play a major role in many biological processes. The activity of the voltage gated sodium channels expressed in neurons and muscles is essential for the action potential in these excitable tissues. In non excitable tissues sodium channels are responsible for receptor potential like the one recorded in taste buds and fluid balance in epithelia such as salivary glands and different part of the renal nephron. Zinc affects both the voltage gated and the epithelial sodium channels.

1.1.1 Voltage Gated Sodium Channels

Horning and Trombley compared the effects of micromolars of Cu²⁺ and Zn²⁺ on voltage-gated conductance in rat olfactory bulb neurons in primary culture. They found that zinc (100 μ M) or copper (30 μ M) inhibited TTX-sensitive sodium and delayed rectifier-type potassium currents but did not prevent the firing of evoked action potentials nor dramatically altered their kinetics [12]. Delgado et al. found that, depending on their concentrations, the divalent metal ions Cu²⁺ and Zn²⁺ presented a differential effect on the kinetic and amplitude of the inward sodium current.

Concentrations of 0.1 μM of these divalent cations produced a slight shift to the left for the I-V curve and an increase in the activation and inactivation rates of the inward current [13]. A somewhat different picture is described by Aedo et al. recording extracellular action potential firing rates of un-dissociated olfactory epithelium neurons. These authors reported that copper and zinc exhibited a biphasic effect on sodium currents. At low concentrations (two-digit nM to one digit μM) they increased the amplitude and speeded up the activation and inactivation kinetics. At higher concentrations, (low μM for Cu^{2+} and two-digit μM for Zn^{2+}) they inhibited the inward sodium currents [14]. Zinc also inhibits the voltage-gated sodium channels in skeletal muscle ($\text{NaV}1.4$) and responds to nerve stimulation by generating action potentials that initiate excitation-contraction coupling.

1.1.2 *Epithelial Sodium Channels*

The amiloride sensitive epithelial sodium channel (ENaC) is physiologically important in tissues regulating fluid balance such as the kidney, where it is important for the regulation of the extracellular fluid volume, and in the lungs, where this channel is instrumental in the maintenance of the appropriate airway surface liquid volume that lines the pulmonary epithelium [15, 16]. In addition the ENaC has been proposed to function as a component of the salt-taste-receptor system [17].

In amphibian A6 cultured cells apical side Zn^{2+} inhibits ENaC. A similar inhibitory effect was observed in Xenopus oocytes expressing $\alpha\beta\gamma\text{rENaC}$ (α -, β -, and γ -subunits of the rat epithelial Na^+ channel) when Zn^{2+} was applied at 1–10 μM . In contrast 100 μM Zn^{2+} led to a stimulatory effect. The stimulatory effect of high Zn^{2+} concentrations could be reproduced by intra-oocyte injection of Zn^{2+} (~10 μM), which had no direct effect on the amiloride-sensitive conductance, but switched the effect of extracellular Zn^{2+} from inhibition to activation. The voltage dependence of this effect in Xenopus oocytes revealed that direct, non-competitive pore block is the main cause of ENaC inhibition [18]. Somewhat contradicting results were reported by Sheng et al. utilizing an oocyte expression system, showing that external Zn^{2+} rapidly and reversibly activates ENaC in a dose-dependent manner with an estimated EC_{50} of 2 μM . External Zn^{2+} in the high Na^+ bath also prevents or reverses Na^+ self-inhibition with similar affinity. Zn^{2+} activation is dependent on extracellular Na^+ concentration and is absent in ENaCs containing γH239 mutations that eliminate Na^+ self-inhibition [19].

1.2 *Potassium Channels*

The potassium (K^+) channel superfamily comprises voltage-gated, inward rectifier, calcium-activated and leak channels. Members of this super family are found throughout most of the eukaryotic kingdom and display the widest range of channel properties. Physiologically, potassium channels are responsible for the resting membrane potential of mammalian cells and are instrumental in the mechanism underlying the secretion of hormones such as insulin and the normal activity of nerves, striated and cardiac muscles, as well as function of the immune cells. Therefore, it is not surprising that mutations in potassium channels were identified as the cause of a number of diseases including cardiac arrhythmia [20], migraine and migraine-related disorders, epilepsy, depression, modulation of the aggressiveness and metastasis of tumors, contribution to the deafness-associated sensitization of the neural auditory pathway [21] as well as the development of certain types of diabetes [22].

1.2.1 Voltage gated potassium channels

K_v1.5: These channels underlie the ultrarapid delayed rectifier current of cardiac and smooth muscle [23]. In the cloned Kv1.5 channel, Zn²⁺ causes channel inhibition resulting in a shift to the right in the half-activation potential ($V_{1/2}$), and substantial slowing of channel activation [24]. External Zn²⁺ causes a substantial depolarizing shift of the midpoint of the charge-voltage ($Q-V$) curve, which is larger than the Zn²⁺-induced shift of the conductance-voltage ($g-V$) relation. It appears that the channel is accessible to Zn²⁺ binding at a number of states within the activation pathway. The second component of the gating charge displacement appears coupled to the blocking action of Zn²⁺[25]. Using site-directed mutagenesis, Kehl et al. addressed the mechanistic basis for the inhibitory effects of external H⁺ and Zn²⁺. They revealed that the binding of H⁺ or Zn²⁺ to histidine residues (H463) in the channel turret is necessary but not sufficient for the inhibitory effect of Zn²⁺, and proposed that protons and Zn²⁺ ions inhibit hK_v1.5 currents by affecting channel availability [26].

K_v1.3: Voltage-activated potassium currents in rat hippocampal neurons contain two components [27]: the slowly-inactivating delayed rectifier current (IDR) and the fast transient current (IA). In addition to the Kv2.1, the Kv1.1, Kv1.2, Kv1.3 and Kv1.6 K_v1.3 contribute to the IDR [28]. The K_v1.3 channels are also expressed abundantly in human T cells [29], where they play an important role in setting the resting membrane potential, cell monogenesis, apoptosis and volume regulation [30, 31]. Application of Zn²⁺ (100 μM-5 mM) to hippocampal neurons reversibly inhibited the recorded currents. The activation midpoint was shifted by about 40 mV (total current) and 30 mV (delayed-rectifier current) towards positive membrane potentials and the activation kinetics were slowed significantly (2 - 3 fold). The current amplitudes were reduced in a concentration-dependent manner to about 0.5 of the control value. The effects of Zn²⁺ were saturated at the concentration of 1 mM and were pH independent (between 6.4 and 7.35) [31]. Zinc at micromolar concentrations blocks the Kv1.3 in T cells. Work by Teisseire et al. suggest that in these cells zinc acts on two independent binding sites; a high affinity site, that saturates at 20μM and a low affinity site which saturates at 100μM [29].

K_v2: These channels are a major component of delayed rectifier potassium currents (IK) in cortical neurons and exist in large, highly phosphorylated clusters on the surface of soma and proximal dendrites [32]. Working with rat primary cortical cultures, Aras et. al found that transient modulation of Kv2.1 activity and localization following ischemia is dependent on a rise in intracellular free Zn²⁺ which plays a critical role in the ischemic modulation of Kv2.1. Zn²⁺chelation, similar to calcineurin inhibition, attenuated ischemic induced changes in K⁺ channel activation kinetics and blocked Kv2.1 declustering. Thus, Zn²⁺ may represent a novel early signal in the modulation of Kv2.1 channel activity and localization following sub-lethal chemical ischemia [32].

K_v4: These channel complexes mediate the neuronal somatodendritic A-type K⁺ current (ISA), playing pivotal roles in dendritic signal integration. Zn²⁺ is found in the T1 domain of the channel which undergoes conformational change in response to oxidative stress induced by reactive nitrogen oxide species (RNOS) derived from nitric oxide (NO) metabolism. The intracellular application of a NO donor (MAHMA-NONOate) to inside-out patches induces rapid inhibition of Kv4.1 channels [33]. To observe this inhibition, it is sufficient and necessary to have two cysteines across the T1 inter-subunit interface (C110 and C132), and intracellular Zn²⁺ antagonizes it [33],

34]. Addition of reducing reagents (reduced glutathione, GSH; or dithiothreitol, DTT) reverses the NO-induced inhibition. Therefore, it was proposed that the inhibition of the channel by nitrosative regulation results from the formation of a disulfide bond between C110 and C132, which straight-jackets the T1-T1 interface. These results suggest the cross-talk between NO and Zn²⁺ [35-37] may also be critical for redox regulation of ISA in the nervous system [34].

KCNQ (Kv7): These proteins comprise a sub-group of the voltage-activated potassium channel family. The family consists of five members (KCNQ1 to 5 also named Kv7.1 to Kv7.5) encoded by single genes, which all give rise to proteins forming slowly activating potassium selective ion channels with currents called the M-current, IM [38, 39]. The M-current is central in the sub-threshold control of neuronal firing due to its unique modulation by neurotransmitters, voltage-sensitivity, and diverse regulatory pathways [40, 41]. The physiological importance of the KCNQ channel family is emphasized by the fact that mutations in four of the five genes have been linked to human pathologies. Zn²⁺ and protons stimulate the channels in a concentration-dependent, reversible, manner with an EC₅₀ value of 21.8 μM at pH 7.4 and an IC₅₀ value of 0.75 μM at pH 6.1. The positive modulatory action of zinc on mKCNQ5 was highly pH-dependent, reaching close to 10-fold potentiation of control levels at pH 8.2 and 30 μM zinc [41]. This report on the stimulatory effect of zinc on M current was in contrary to studies by Robinson et al. working with differentiated NG108-15 mouse neuroblastoma-rat glioma hybrid cells showing inhibition of the M current by various divalent cations, zinc being the most effective, with IC₅₀ of about 10μM[42]. However, since neuroblastoma-rat glioma hybrid cells also expresses KCNQ2, 3, and 5 [43, 44] as well as merg1a currents [45], the reported inhibitory effect observed in the NG108-15 cell line could reflect a heteromeric assembly of ion channels [41]. A recent publication identified Zinc-pyrithione (ZnPy) as a new KCNQ channel opener, which potently activates KCNQ2, KCNQ4, and KCNQ5 causing both a hyperpolarizing shift in the G-V curve and a marked increase in overall current amplitude, with an EC₅₀ value of 1.5 μM for KCNQ2. The ZnPy effects on the important cardiac KCNQ1 potassium channels remain largely unknown [46-48].

1.2.2 Potassium "Leak" Channels;

Only in the mid 90s of the previous century leak potassium channels, such as a TWIK (tandem of pore domains in a weak inward rectifying K⁺ channel, now called TWIK-1), were identified. This finding resulted in the rapid discovery of a new family of mammalian background potassium channels [49, 50]. This family of cloned mammalian background K⁺ channels embraced 14 additional members encoded by different genes. The members were divided into six subfamilies (TWIK, TREK, TASK, TALK, THIK, and TRESK) on the basis of sequence similarity and functional resemblance [51, 52], some of which are modulated by zinc.

TREK: These channels generate outwardly rectifying currents that are increased by unsaturated fatty acids such as arachidonic acid and mechanical stimuli such as cell swelling, stretch, and positive and negative pressure [53, 54]. TREK-1 is potently modulated by both copper and zinc. Zinc inhibits the channel with an IC₅₀ for inhibition of about 659 μM [55]. TREK-2 currents were differentially affected by metallic ions; there was potent inhibition by Pb²⁺, dose dependent stimulation by Zn²⁺, and little effect of the other metal ions. Kim et al. revealed that the structural elements in TREK-2 responsible for its Zn²⁺-mediated enhancement are localized to the first

pore and the preceding extracellular loop of TREK-2, where multiple residues, including His121, His156, Asp158, and Asn177, are likely to be involved in the zinc activation effect [56].

TASK: The TASK (TASK1, TASK3, and TASK5) subfamily of K₂P channels is regulated by a number of different pharmacological and physiological mediators. At pH 7.4 TASK3 channels are selectively blocked by zinc in a manner that is both pH_o- and [K]_o-dependent. Testing acutely dissociated, capsaicin insensitive nociceptive neurons from rat dorsal root ganglion (DRG) Cooper et al. showed that when acidic solutions were used, currents that were insensitive to Ba²⁺ and Ruthenium Red were inhibited by Zn²⁺. This Zn²⁺ sensitive component of the proton sensitive current was consistent with TASK-1. In contrast, Zn²⁺ produced substantial changes in excitability at physiological pH [57]. Mutation of both the Glu-70 residue in the M1P1 loop and the His-98 residue in the pore region abolished the block, suggesting that the two residues may contribute to a zinc binding site [58]. TASK-3 is about 50 fold more sensitive to zinc inhibition than TREK-1 with an IC₅₀ for inhibition of about 12.7 μM [55]. The amino acids His98, which acts as a proton sensor [59], and Glu70, which is required for inhibition by ruthenium red [60], are critical for the zinc inhibition of TASK-3[61].

TRESK: Tresk (TWIK-related spinal cord K⁺ channel) is a two-pore-domain potassium (2PK⁺) channel regulated by the calcium/calmodulin-dependent protein phosphatase calcineurin. Utilizing a Xenopus oocyte expression system, Czirjak and Enyedi showed that zinc and mercuric ions inhibited TRESK with IC₅₀ values lower than 10μM. In the mouse Zn²⁺ and Hg²⁺ exerted a significantly stronger inhibitory effect on TRESK than on the other TALK-1, TASK-1, TASK-2, TASK-3, THIK-1, TRAAK, TREK-1, and TREK-2 channels [62]. It is worth noting however that zinc that inhibited the TRESK channel in rodent in the low micromolar range was without effect on human TRESK [63]. It is postulated that the His132 in the mouse TRESK was partly responsible for this difference [62].

1.2.3 K_{ATP} Channel

The K_{ATP} channel is a large macromolecular complex in which four inwardly rectifying potassium channel (Kir6.x) subunits form a central pore surrounded by four regulatory sulphonylurea receptor (SUR) subunits [64-66]. Physiologically, the K_{ATP} channel serves as a metabolic sensor, coupling cellular metabolism to electrical activity in a wide range of tissues. Opening of K_{ATP} channels under conditions of low metabolism leads to membrane hyperpolarization and switches off cellular functions. Metabolic sensitivity varies between K_{ATP} channels: cardiac and skeletal K_{ATP} channels open only when metabolic stress is severe (as in ischemia) while β-cell and neuronal channels open when plasma glucose levels fall [66]. In contrast to β-cells, isolated α-cells are poorly responsive to glucose that slightly lowers intracellular calcium without significantly affecting cell metabolism or K_{ATP} channel activity [67]. In the CNS (central nervous system) K_{ATP} are present at high concentrations in mossy fibers [68-70]. When the cytoplasmic ATP/ADP ratio rises, K_{ATP} channels close and cells depolarize [71]. Zinc activates K_{ATP} channels, leading to cell hyperpolarization [71, 72]. This activation by zinc and the inhibition of the voltage gated calcium channels contribute to the observed decrease in synaptic activity in the presence of zinc released during tetanic stimulation [69]. The action of zinc on K_{ATP} channels occurs at lower zinc concentrations compared to its inhibition of the voltage dependent calcium

channels (about 10 μ M). Zinc at concentrations near 20 μ M, significantly enhances potassium current through K_{ATP} channels [69].

1.2.4 Ca^{2+} Activated K Channels (BK Channels)

Large-conductance voltage- and Ca²⁺-activated K₊ (BK_{Ca}, Slo1 BK or KCa1.1) BK channels are found in neurons [73-75], chromaffin cells [76-78], inner hair cells of cochlea [[79-81], and skeletal [82, 83] and smooth muscles [84, 85]. These channels are activated by membrane depolarization and elevation of intracellular calcium concentration [75]. Functionally BK channels participate in many crucial physiological phenomena including vaso-regulation, synaptic transmission, and hormone secretion, mainly by affecting membrane excitability [86]. In an heterologous expression system the hyperpolarization-activated currents in oocytes were sensitive to extracellular divalent cations, significantly blocked by 10 μ M Zn²⁺ [87]. The concomitant increases in intracellular Ca²⁺ and Zn²⁺ in ischemia/ hypoxia and the cytoprotective role of the BK channel under the pathological conditions prompted examination as to whether Zn²⁺ is also a physiological activator of the channel. The BK channel indeed contains multiple putative Zn²⁺ -binding amino acid sequences such as HXXXH (X represent any amino acid). Hou et al. utilized excised patch clamp measurements from heterologously expressed human Slo1 (hSlo1)3 BK channels revealing that intracellular Zn²⁺ robustly activates the channel and that a mutation of one histidine residue in the RCK1 domain fully abolished the stimulatory effect of Zn²⁺. Their results therefore suggest that Slo1 coordinates Zn²⁺ using amino acid ligands in the RCK1 domain and that the Slo1 BK channel is a positive effector of intracellular Zn²⁺ signaling [88].

1.2.5 Other K_i

Several lines of evidence suggest that excessive K⁺ efflux and intracellular K⁺ depletion are key early steps in the early phases of apoptosis [89]. Recent evidence indicates that activation of caspase-3 is in part responsible for the induction of apoptosis in zinc-treated HL-60 cells and human peripheral blood lymphocytes [90] (see chapter 5). Recently, Shi et al. used the MES23.5 cell line, derived from somatic cell fusion of rat embryonic mesencephalon cells, and the murine neuroblastoma-glioma cell line N18TG2, showing that exposure of these cells for 24 hours to 60 μ M zinc led to augmentation of tetraethylammonium (TEA) sensitive potassium efflux which led to the activation of caspase-3. This finding is consistent with zinc induced apoptosis by facilitating the opening of K⁺ channels [91]. Another potassium channel affected by zinc is the human *Ether-à-go-go* Related Gene (hERG) investigated by at least two groups. In contrast to other divalent ions such as Cd²⁺, Ni²⁺, Co²⁺ or Mn²⁺, Zn²⁺ decreased the current amplitude without shifting the activation on the voltage axis [92, 93].

1.3 Hydrogen Channels

The Hydrogen-dependent proton channel (Hv1) is activated by membrane depolarization, an alkaline extracellular environment, endocannabinoid anandamide, and removal of extracellular zinc which is a potent inhibitor of this channel [94]. Acid extrusion by proton channels is required for example during histamine secretion by human basophils [95]. Zn²⁺ inhibits histamine-stimulated acid secretion by airway

epithelial cells [96-98]. A contribution of hydrogen channels to cell volume regulation has been proposed in microglia [96, 99] and chondrocytes [100, 101]. Facilitation of reactive oxygen species (ROS) production by DUOX1 in airways, analogous to in phagocytes, has been suggested [96, 102]. Proton channels are highly expressed in human B cells [96] where they participate in B-cell receptor-mediated signaling cascades and antibody responses, most likely by enabling ROS production [103]. Possible facilitation of CO₂ elimination by the lung has been hypothesized [104]. A contribution to pH and membrane potential regulation in cardiac fibroblasts, especially during ischemia, has been proposed [96, 105]. In addition the channel plays a role during alkalization of the sperm cytoplasm which leads to its activation after it is introduced into the female reproductive tract [94]. Zn²⁺ is among the most potent inhibitors of voltage-gated proton channels, inhibiting the channels in the low micromolar range [96, 106-108]. Unlike traditional ion channel blockers, Zn²⁺ does not occlude the channel, but instead binds to the external surface of the molecule where it slows channel opening and shifts the voltage dependence positively [109]. The efficacy of Zn²⁺ in inhibiting the proton channel is greatly reduced at low extracellular pH, and the details of the competition between H⁺ and Zn²⁺ could be explained only by assuming that the external Zn²⁺ binding site comprised 2 or 3 (but not 1) titratable groups with pKa 6.2–7.0, suggesting His residues [110]. The human proton channel turns out to have two His residues that are accessible to the external solution, and their mutation individually to Ala lowers Zn²⁺ sensitivity by an order of magnitude; the double His→Ala mutant has only weak Zn²⁺ sensitivity [106, 108]. The internal Zn²⁺ binding site has not been identified. Applied intracellularly, Zn²⁺ has less dramatic effects that are qualitatively consistent with surface charge effects, namely shifting the g H–V relationship negatively, slowing tail current decay (larger τ tail) and decreasing the limiting g H, g H,max [106, 110, 111].

2. Divalent Channels

Voltage and ligand gated calcium channels, purinergic receptor activated channels as well as the various type of Transient receptor potential (TRP) channels are all regulated by zinc. Some of these channels are zinc permeable which leads to the channel inhibition. In other channels zinc binds to specific sites which modulate channel activity. Due to limited space we will discuss only the effect of zinc on the calcium channels (voltage and ligand gated), P2X channels and members of the TRP channels.

2.1 Voltage Activated Calcium Channels

Voltage-gated Ca²⁺ channels mediate Ca²⁺ entry into cells in response to membrane depolarization. Electrophysiological studies reveal different Ca²⁺ currents divided into high (L, N-, P/Q-, R- type; HVA), and low voltage (T type) activating channels (LVA). These channels were later clustered into three groups according to their α_1 subunit [112]. Members of Ca_v1 family (L-type) are involved in initiating muscle contraction, endocrine secretion, and gene transcription. Members of the Ca_v2 (N, P/Q and R) family are involved in the initiation of rapid synaptic transmission. The Ca_v3 family (T-type) participate in the depolarizing potentials and repetitive burst firing in dorsal root ganglions [113, 114], in neuronal activity during rapid eye movement [113, 115] as well as in pain signaling [116]. Recent publications substantiated the role of T-Type

Calcium Channels (TTCC) not only in neuronal firing but also in hormone secretion and cardiac pathology [117-120]. Measuring single dihydropyridine-sensitive Ca^{2+} channels Wingar et al. report that Zn^{2+} produces "flickery" block of the open channel. It was suggested that Zn^{2+} blocks the channel by binding to a site closer to the surface membrane than the Ca^{2+} binding site [121]. Zn^{2+} permeates these channels under ionic conditions normally present in the brain thus leading to channel inhibition under physiological conditions. In addition extracellular acidity enhanced the permeation of Zn^{2+} relative to Ca^{2+} through voltage gated Ca^{2+} channels [122]. Under normal Ca^{2+} concentrations the HVA channels are inhibited by zinc with the IC_{50} of between 7 μM and 30 μM [123, 124]. According to Sun et al. the sensitivity of HLA and LAV channels to Zn^{2+} was approximately ranked as $\text{Cav1.2} > \text{Cav3.2} > \text{Cav2.3} > \text{Cav2.2} > \text{Cav2.1} \geq \text{Cav3.3} = \text{Cav3.1}$ supporting the notion that the Zn^{2+} block, mediated by multiple mechanisms, may depend on conformational changes surrounding the $\alpha 1$ pore regions [123]. As [125] zinc differentially regulates the three Ca_v3 channel isoforms: it preferentially inhibits Cav3.2 channels with an IC_{50} in the submicromolar range (<0.8 μM), which is 100 and 200-fold lower than what is observed for Cav3.1 and Cav3.3 channels, respectively [120, 123]. In addition, zinc significantly slows the deactivation kinetics of the Cav3.3/ T-current, i.e. the tail current, which consequently causes an enhanced calcium entry through Cav3.3 channels [120]. Under these experimental conditions, it appears that zinc operates as a mixed blocker/opener of Cav3.3 channels. Zinc produces its blocking effect on Cav3.2 channels through binding to an extracellular histidine (His191) residue localized in the S3–S4 segment of domain I of Cav3.2 channel [126]. His191 was originally identified as a critical determinant of the nickel block of Cav3.2 channels [127]. In addition, the oxidizing agent, ascorbate, also produces Cav3.2 channel inhibition through the metal-catalyzed oxidation of this specific His191 [128], further demonstrating that this amino-acid is an important checkpoint in the modulation of Cav3.2 channel activity.

2.2 Glutamate Sensitive Channels

There is consensus that glutamate is the main excitatory brain transmitter. Two major families of membrane ionic channels are activated by glutamate: the NMDA and the AMPA/kainate receptors. All receptors are permeable to sodium and in varying degrees also to calcium [129]. The NMDA receptor is an oligomeric cation channel which mediates physiological and pathological processes such as long-term potentiation (LTP), synaptic plasticity and neurodegeneration via conditional Ca^{2+} signaling [130, 131, 132]. The ionic influx through the open NMDA channel pore is consequent to the presynaptic release of glutamate and postsynaptic membrane depolarization, which relieves voltage-dependent Mg^{2+} block [132]. Impairment of the Mg^{2+} block of NMDA receptors can lead to excessive Ca^{2+} influx into neurons and the generation of nitric oxide and/or reactive oxygen species. The AMPA and kainate classes of glutamate receptors belong to the same superfamily as the NMDARs and share approximately 25% homology. AMPA receptors (AMPARs) are made up of a combination of four subunits (GluR1–4) and require only glutamate application for activation. Physiologically, AMPARs are thought to regulate the fast excitation required to remove the magnesium block of nearby NMDARs [133]. Kainate receptors are involved in modulating synaptic transmission and plasticity, particularly in the hippocampal formation, and are also involved in diseases such as schizophrenia and

major depression [134]. Twenty years ago, a first study demonstrated that zinc permeates, as well as potently modulates, both AMPA and NMDA glutamate-gated currents at either type of receptors [129]. The action of zinc at the NMDA receptor is apparently related to the metal interaction with two separate mechanism(s), each attributed to different metal binding sites on the receptor subunits that form the NMDA ionic channel [129, 135, 136]. Zinc inhibits NMDA evoked currents, acting at a voltage independent site on the NR2A subunit and at a voltage dependent site on the NR2B subunit [129, 137]. By contrast, the action of zinc at the AMPA/kainate receptors is biphasic, while micromolar concentrations increase the receptor activity; millimolar concentrations decrease it [138]. Minor variations to this general picture have recently emerged in view of the description of a zinc-insensitive population of AMPA/kainate receptors, a finding possibly attributed to the varying subunit composition expressed by individual receptors [129].

2.3 TRP channels

Transient receptor potential (TRP) channels are polymodal cellular sensors involved in a wide variety of cellular processes, mainly by changing membrane voltage and increasing cellular Ca^{2+} . TRP channel superfamily consists of 28 nonselective cation channels that are subdivided into six subfamilies according to the homology of their amino acids and structures [139, 140]. TRP channels are ubiquitously expressed in many cell types and are functionally diverse. They act as the principal sensory transducers for a wide range of physical and chemical stimuli [141]. TRP channels mediate responses to light, nerve growth factors, pheromones, olfaction, taste, mechanical changes, temperature, pH, osmolarity, vasorelaxation of blood vessels, and metabolic stress [139, 140]. Recently, the functional characteristics of TRP channels in several disease states have been examined and their roles have been revealed to be much broader than classical sensory transduction. To this end it was documented that TRP channels are involved in various human diseases such as cardiovascular diseases [142], hypertension [143], endothelial dysfunction [144], metabolic syndrome (MS) [145], kidney diseases [146], and metabolic diseases [147].

The TRPM7/ChaK1 is a unique channel/kinase that contains a TRPM channel domain with 6 transmembrane segments fused to a novel serine-threonine kinase domain at its C terminus. The kinase domain is essential for the functional expression of the channel. The kinase activity is decreased by Zn^{2+} , while channel activity is inhibited by zinc as well as by calcium and magnesium ions [148, 149]. Recently Martineau et al. showed that Ca^{2+} , Mg^{2+} , Zn^{2+} or Gd^{2+} decreased ^{109}Cd cytotoxicity in MC3T3-E1 osteoblasts cells suggesting the involvement of non-selective cationic channels. The Mg^{2+} -sensitive part of $^{109}\text{Cd}^{2+}$ uptake increased at acidic pH, a condition known to stimulate TRPM7 channel activity. In contrast to Mg^{2+} , Zn^{2+} -induced inhibition was observed at any given pH. These results show the possible involvement of different mechanisms of transporting Cd^{2+} uptake at basic pH, which is sensitive to Zn^{2+} inhibition but insensitive to Mg^{2+} . The results reveal saturable mechanism of transport for Cd^{2+} in MC3T3-E1 cells. Transport properties are in many ways similar to TRPM7-mediated uptake including inhibition by Ca^{2+} , Mg^{2+} , Zn^{2+} and Gd^{2+} cations, as well as by pharmacological agents such as 2-APB and carvacrol [150]. Abnormal lysosomes and mitochondria are common features of the human lysosomal storage disorder known as mucolipidosis IV (MLIV) and caused by the loss of TRPML1 ion channel function. Recently, Eichelsdoerfer et al. showed that the loss of TRPML1

function results in intracellular chelatable zinc dyshomeostasis. They proposed that chelatable zinc accumulation in large lysosomes and membranous vacuoles may contribute to the pathogenesis of the disease and progressive cell degeneration in MLIV patients [151]. T1R3-TRPM5 and TRPV are involved in the Complex tasting divalent salts (CTDS) that evoke metallic, bitter, salty, and astringent sensations include the divalent salts of iron, zinc, copper, and magnesium. Riera et al. showed recently that at low concentrations, compounds such as FeSO₄ and ZnSO₄ stimulate the gustatory system through the hedonically positive T1R3–TRPM5 pathway, and at higher concentrations, their aversion is mediated, in part, by the activation of TRPV1 [152].

2.4 P2X Receptor Channels

The effect of zinc on the P2X receptor was comprehensively reviewed by Huidobro-Toro et al. in 2008 [129]. P2 receptors can be subdivided into P2X and P2Y families, based on their actions and signaling mechanisms: P2X operate as ligand-gated ion channels and P2Y are G-protein-coupled receptors. P2X receptor channels open upon binding of extracellular ATP and permeate cations including Ca²⁺, resulting in membrane depolarization and/or an elevation in intracellular Ca²⁺ concentration. There are seven mammalian P2X subunits, P2X1-P2X7, which assemble homo/hetero-trimers to form functional P2X receptors [153]. A role for P2X receptor activity was implicated in urinary voiding, platelet aggregation, vasodilatation, fertility and bronchial airway inflammation [129]. In addition these channels are involved in synaptic transmission, taste sensation and control of smooth muscle [129, 154]. The subtypes P2X4 and P2X7 are also critically involved in pain and inflammation [154]. There are two main effects of copper and zinc ions on the channels; either potentiation or inhibition of the ATP-evoked currents, effects observed in concentrations ranging from 0.3 to 100 μM. For example, while zinc inhibited in a concentration dependent manner the P2X1 receptor activity [155], zinc or copper potentiated the P2X2 receptor activity [129, 156]. The P2X3 receptor is also potentiated by zinc [155]. However, contrary to the observations in the P2X2 or P2X3 receptors, both zinc and copper inhibit the P2X7 receptor-gated currents [153]. An exceptionally interesting case is the P2X4 receptor since this purinoceptor is potentiated by zinc but inhibited by copper [129, 157]. Zinc or copper acting at concentrations within 1–10 μM, notoriously modify P2X receptor evoked currents [129, 156, 157 , 158-160], an indication that these receptors may be persistently modulated by the basal concentration of trace metals permanently found in neuronal synapse. The facilitator mode of zinc action in the P2X2 and P2X4 receptors is due to a reversible and parallel leftward displacement of the ATP concentration-response curve [156]. The zinc mode of action suggests that the binding of this metal to the facilitator site increases the receptor affinity for ATP [129, 157, 160]. The histidine residues, His-120 and His-213 were reported as playing a key role for the modulator action of both zinc and copper potentiation; the single substitution of each of these two histidine residues rendered the respective receptor mutants resistant to the action of both metals altogether [156, 161] while His-140 was involved in the inhibition of the P2X4 [160]. P2X receptor was also described as a zinc sensing receptor acting as a Ca²⁺ entry channel that is markedly sensitive to changes in external H⁺ and Na⁺ concentration and is gated by zinc alone, ATP alone, or both. [162-165].

3. Conclusion and Perspectives

It is clear that many channels are regulated by zinc, which either enhances or decreases their activity due to exposure to an increase in extracellular or elevation of intracellular zinc. In many cases zinc may serve as part of a feedback mechanism and its secretion leads to a reduction in the secretion process. Such may be the case in β cells in the pancreas where zinc secreted with insulin blocks L type calcium channels, the activation of which trigger depolarization and secretion of insulin and zinc. Zinc may also act indirectly by stimulating the expression of channel regulating proteins. An example of this mechanism is found in the stimulation of ZnT-1 expression which consequently inhibits the L type calcium channel due to reduction of its alpha subunit at the plasma membrane [166-169]. Exposing cardiomyocytes to sub-lethal concentrations of zinc for only 2 hours will lead to long lasting augmentation of ZnT-1 that can be measured 24 and 48 hours thereafter. Studies of this long term indirect effect of zinc as channel modulator is expected to assist in elucidating yet unexplored ways by which zinc modulates channels in physiological as well as in pathological conditions.

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8. Zinc Transporters

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Abstract. Zinc transporters maintain zinc homeostasis in integrative systems through controlling absorption and excretion of this essential micronutrient as well as by regulating inter-organ and intracellular zinc for functional needs. Studies of the 24 zinc transporters derived from two families have relevance to both clinical science and basic science. These studies are necessary to address the questions of what transported zinc does and why multiple zinc transporters have evolved.

Keywords. Signal transduction, Metal-response element, Phosphatase, Zinc metabolism

Introduction

The road that has led to our current understanding of mammalian zinc transporters could arguably have originated in studies on metal resistance/tolerance genes identified in microorganisms [1]. Others in the field could emphasize that development of the principles of bioinorganic chemistry were necessary before an understanding of how cells transport zinc ions could be achieved [2]. The early application of radioactive zinc to integrative and single cell systems made it clear that while biological systems maintain stringent control of zinc concentrations, this is accomplished through mechanisms that regulate zinc fluxes into and out of these systems [3-5].

In contrast to the slow pace of this evolution in understanding zinc transport, the last 15 years has seen rapid development. A seminal contribution was the cloning of the first mammalian zinc transporter [6]. This has led to an understanding of the basic contributions of zinc transporters to maintaining zinc homeostasis in integrative systems through controlling absorption and excretion of this essential micronutrient as well as how inter-organ and intracellular zinc is regulated for functional needs [7, 8]. Studies on zinc transporters have relevance to both clinical science and basic science and the bridges that connect these areas of investigation. These studies address questions that range from the electrophysiology of the actual transport process to those asking questions about what does the zinc do after it is transported.

1. Zinc Transporter Families

While zinc may enter cells by numerous mechanisms including co-transport as a complex or transporters/channels for other substrates [9] (see also chapter 7), there is

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general consensus that proteins arising from two gene families are the major players in this regard [10]. Null mutations of a number of these genes produce embryonic lethality,

which clearly addresses their critical importance to zinc transport and health. For this review all capital letters will be used to refer to the protein, e.g. ZIP1 where as Zip1 will refer to the mRNA and italic Zip1 will identify the gene.

2. ZnT (SLC30) Family

ZnT (solute-linked carrier 30) proteins act to lower intracellular zinc concentrations either through cellular export or by import into a cellular compartment or organelle (Figure 1).

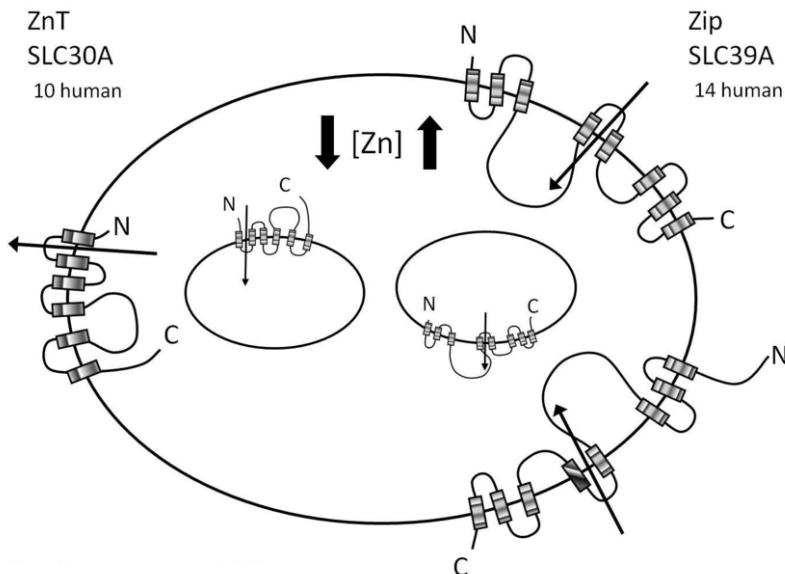


Figure 1. A generalized cell showing the localization of the ZnT and ZIP transporter proteins. The ZnT proteins have six transmembrane domains and generate zinc efflux out of cells or into organelles or vesicles. The ZIP proteins have eight transmembrane domains and generate zinc influx into cells or from organelles or vesicles. (Modified from J. Liuzzi and R. Cousins [11]).

There are three subfamilies in this group with subfamilies II and III found in mammals. These are predicted to have six trans-membrane domains (TMDs) and cytoplasmic amino and carboxy termini [12]. ZnT proteins also share a histidine-rich loop between TMDs IV and V which likely places this potential metal-binding site in an intracellular location [12]. These may serve as stabilizing domains for zinc prior to movement through the channel formed by the TMDs. Phylogenetic relationships among the homologs of the ZnT family are shown in Figure 2.

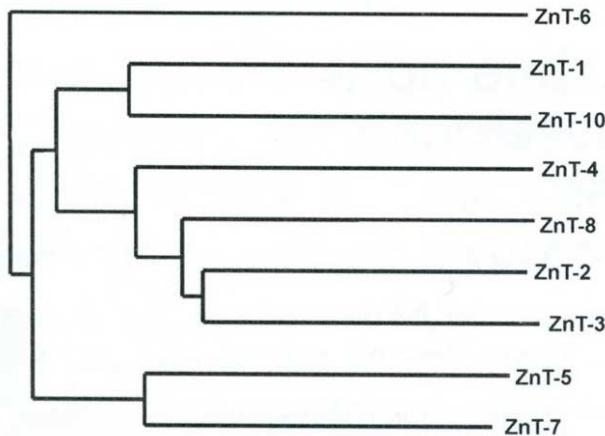


Figure 2. Dendrogram showing the phylogenetic relationships among the ten homologs of the ZnT family. From M. Seve et al. [13].

2.1. *ZnT1* (*SLC30A1*)

ZnT1 was the first mammalian zinc transporter to be identified and characterized [6]. Mutation of zinc-sensitive BHK cells to produce zinc resistance was used for the isolation. *ZnT1* is mapped to chromosome 1 in both humans and mice. ZnT1 is ubiquitously expressed in tissues. Localization is primarily to the plasma membrane, but vesicular, perhaps endosomal, is frequently observed as punctate staining [6, 14, 15]. A basolateral membrane location has been identified in polarized cells, specifically rat enterocytes and cells lining the thick ascending and distal convoluted renal tubules [14, 16]. These findings suggest ZnT1 has a major role in zinc enteric absorption and renal reabsorption. Acinar cells of the pancreas produce appreciable ZnT1 which is located primarily at the apical membrane [17], supporting a role in zinc excretion via that organ. ZnT1 is highly expressed in the placenta suggesting a transport function to support fetal development [16, 18, 19]. Supporting this role is that homozygous knockout of *ZnT1* produces early embryonic lethality [20].

Regulation studies have shown that *ZnT1* exhibits responsiveness to zinc. This follows the zinc sensitivity of *ZnT1* shown during initial characterization where a MTF-1 regulated mechanism was proposed [6]. Homozygous knockout of *Mtf-1* leads to a reduction in ZnT1 mRNA levels in visceral yolk sac, supporting a role for MTF1 in *ZnT1* regulation [18]. Responsiveness of *ZnT1* to the dietary zinc supply has been demonstrated in rodents and humans [14, 17-23]. These responses show profound tissue and species specificity as well as differences in response to zinc restriction versus zinc supplementation. For example, intestinal ZnT1 mRNA levels at steady state are refractory to zinc restriction of rats [14, 22], while in mice pancreatic and peripheral blood mononuclear cells (PBMC) ZnT1 mRNA is markedly down-regulated when a zinc-restricted diet is provided [17, 22]. ZnT1 protein levels of mature red blood cell membranes are reduced in mice during dietary zinc restriction [24]. ZnT1 may provide a protective role in central nervous system. There is an increase in ZnT1 in mouse forebrain during development [25] and ZnT1-induction in gerbil forebrain with transient ischemia and rat astroglial cells is viewed as protective [26, 27]. These

findings are in line with demonstrated reduction in zinc toxicity via *ZnT1* transfection [15, 28]. Studies on ZnT1 and tumor development have produced mixed results.

ZnT1 may also have a role in cell signaling. Of note is that ZnT1 expression is uniformly decreased in human primary monocytes, T-cells and granulocytes upon activation [21]. ZnT1 has been shown to inhibit the L-type-calcium channels (LTCC) at least in neuronal cells [27, 28]. This occurs through interaction of ZnT1 with LTCC [29]. In *Caenorhabditis elegans*, CDF1 (cation diffusion facilitator), an ortholog of ZnT1, reduces intracellular zinc and increases Ras-mediated signaling through inhibition by zinc of Ras and Raf pathways [30, 31]. We proposed that ZnT1 has similar control functions in mammals and could have influences on signaling pathways [32]. The embryonic lethality of the *ZnT1*^{-/-} mutation in mice [20] supports an important role for ZnT1 in zinc-mediated signaling pathway control.

2.2. *ZnT2* (*SLC30A2*)

As with ZnT1, ZnT2 was isolated from a rat kidney cDNA expression library through selection of a zinc-sensitive BHK cell line [33]. These cells accumulate high levels of zinc in vesicles. This characteristic appears to be a key feature of ZnT2-related Zn functions. Modest regulation by dietary zinc and by select hormones are also signatures of ZnT2.

ZnT2 is up-regulated in rat intestine during late gestation and early lactation [19]. High dietary zinc modestly up-regulates ZnT2. ZnT2 transcripts are found in intestines, kidney, placenta, mammary gland, prostate, pancreas, testes, and seminal vesicles and other rodent tissues [reviewed in 10]. There is a clear specificity for ZnT2 expression in secretory tissues. Dietary zinc restriction in mice produces marked reduction in ZnT2 expression in small intestine and pancreatic acinar cells. ZnT2 has a vesicular location in acinar cells of mice fed a zinc-inadequate diet which is virtually undetectable in zinc-restricted mice [22]. Repletion with a zinc-adequate diet restores ZnT2 mRNA levels to normal in 24 hr. Murine *ZnT2* has one consensus metals response element (MRE) binding site that luciferase reporter constructs demonstrate is functional [17]. ZnT2 is localized to zymogen granules of murine pancreas. ZnT2 siRNA knockdown yields zinc retention and decreased ⁶⁵Zn release from the granules [17]. Of note is that ZnT2 in mammary gland shows apical localization [34] and decreases throughout lactation [19, 34]. Mutations in *hZnT2* producing a His to Arg substitution have been shown to produce a neonatal zinc deficiency through decrease in ZnT2 transport capacity that decreases the zinc content of milk [35]. Zinc supplementation reverses the effects of deficiency. Pancreatic ZnT2 expression is proposed to function for zinc release into the gastrointestinal tract for homeostasis [17].

Hormonal regulation of *ZnT2* is a feature that has received considerable attention. Prolactin up-regulates *ZnT2* in prostate and mammary gland [36, 37], testosterone regulates prostatic ZnT2 [36] and glucocorticoid regulates pancreatic ZnT2 [17]. For mammary gland and pancreas hormonal responsiveness has been shown to be mediated by STAT5 signaling using multiple lines of evidence. It is clear that tissue-specific receptors produce ligand stimulated activation of the STAT5 response elements of *ZnT2* [17, 37]. This mode of regulation via STAT5 is not a common mode of hormonal regulation. Details are shown in Figure 3.

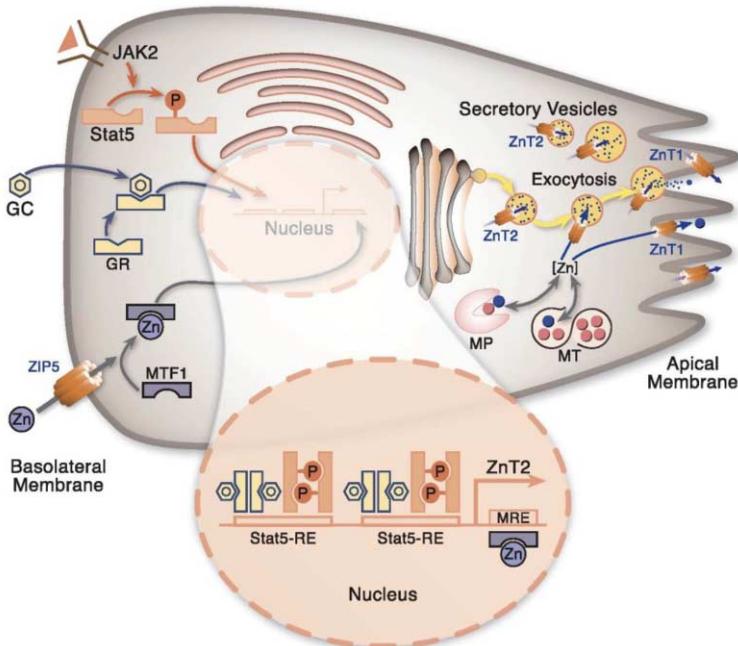


Figure 3. Proposed model for zinc transport and secretion by pancreatic acinar cells. Zinc enters from the peripheral circulation via influx mediated by ZIP5 located at the basolateral plasma membrane. Zinc restriction leads to ZIP5 degradation. Zinc for export includes that which is incorporated into metalloenzymes, metallothionein, or made available for incorporation into zymogen secretory vesicles. Zinc restriction reduces ZnT2 synthesis and zinc transport into vesicles. Glucocorticoid hormones also effect ZnT2 expression via STAT5 signaling which represents one of the few examples of a hormonally responsive zinc transporter gene. Restriction also reduces ZnT1 expression which is located at the apical membrane. Exocytosis via ZnT2, efflux via ZnT1, and ZIP5 influx account for most of the control of pancreatic release of zinc into the gastrointestinal tract. From L. Guo et al. [17]. Reproduced with permission of the National Academy of Sciences.

ZnT2-stimulated accumulation of zinc into lysosomes of fibroblasts via overexpression has been shown [38]. It was suggested that ZnT2-directed zinc is involved in protein trafficking to late endosomes and lysosomes. These findings may integrate zinc trafficking with production and secretion of zinc metalloenzymes.

2.3. ZnT3 (*SLC30A3*)

ZnT3 has a 52% amino acid homology with ZnT2. This led to its identification using library screening [39]. ZnT3 transcripts are limited to the brain, with high abundance in the hippocampus and cortex. These are regions that contain substantial amounts of zinc as detected histologically with Timm's stain and where ZnT3 is localized to synaptic vesicles [40], where between 5–15% of the zinc in brain is found [41]. ZnT3 is also in the ependymal lining separating the cerebrospinal fluid and nervous tissue [42].

Homozygous ZnT3 knockout mice (*ZnT3^{-/-}*) have very little detectable zinc in synaptic vesicles compared to wild type mice [43]. *ZnT3^{+/+}* mice have an intermediate level of zinc packaged into synaptic vesicles compared to homozygous cohorts. These results suggest zinc uptake into synaptic vesicles is through a ZnT3-mediated process

at the vesicle membrane. *ZnT3*^{-/-} mice have less insoluble amyloid plaque accumulation compared to *ZnT3*^{+/+} mice when crossbred with Swedish mutant APP mice, a model for Alzheimer's disease [44]. This suggests zinc is involved in the pathogenesis of the disease (see chapter 21 for further details).

2.4. *ZnT4* (*SLC30A4*)

ZnT4 was discovered shortly after *ZnT2* and was called *Dri42* [45]. *ZnT4* was identified through cDNA library screening and characterized as a novel gene up-regulated in development of rat intestinal mucosa [46]. Expression is high in mammary gland, brain, villus yolk sac, as well as intestinal epithelial cells [19, 46-48]. Localization studies tend to support a vesicular and/or endosomal-like structures in rat intestine. *ZnT4* expression increases in intestine as neonates develop with abundance increasing at the basolateral membrane region of these polarized cells [19, 46]. *ZnT4* has been detected in the trans-golgi network (TGN), consistent with a vesicular secretory function. *ZnT4* is regulated by dietary zinc in mouse intestine [22].

The autosomal recessive mutation in mice that produces the lethal milk (*lm*) phenotype [49] and leads to a decrease in zinc of milk secreted from the mammary gland [50, 51] is caused by a C to T point mutation in *ZnT4* [47]. Defective zinc secretion into milk produces a zinc deficiency in suckling pups that is lethal. Zinc supplementation of the lactating dams or giving zinc to the pups corrects the *lm* phenotype [49]. Reduced zinc secretion into milk occurs in humans and has been attributed to *ZnT2* [35]. Analysis of *hZnT4* expression indicates that *ZnT4* is not responsible for a mammary zinc secretion disorder in humans [52].

ZnT4 down-regulation is associated with prostate disease and prostate cancer [53, 54], and numerous microarray screens of human disorders. In contrast to *ZIP4*, functions related to zinc secretion and intestinal transport of *ZnT4* has received only limited attention. An exception is that mast cells have high levels of *ZnT4* localized to granules which may control zinc pools that regulate activation of procaspase-3 and nuclear translocation of NF- κ B [55].

2.5. *ZnT5* (*SLC30A5*)

Human *ZnT5* was identified from homology to yeast ZRC1 [56]. Atypical for a *ZnT* protein, *ZnT5* has 15 TMDs. Nearly simultaneously *hZnT5* was also cloned by another group and designated *hZTL1*, but subsequently redefined as *hZnT5* [57]. Expression of *ZnT5* is ubiquitous, but has high expression in pancreatic β cells, kidney, brain, parietal cells of the stomach and small intestine [57, 58]. A null mutation of *ZnT5* in mice produces a phenotype characterized by poor growth, abnormal bone development, weight loss, and male-specific cardiac arrhythmias [59].

ZnT5 interacts with *ZnT6* to form a complex that transports zinc into a secretory pathway [60]. Both are located in the TGN [56, 61]. The complex considered essential to activate tissue-nonspecific alkaline phosphatase (TNAP) [62]. These results are the first demonstration that zinc transporter activity is required for zinc-transfer for the purpose of producing an active holoenzyme.

Two major *ZnT5* transcripts are produced [56, 57]. They differ at the 5' and 3' ends with variant B being a shorter transcript [56, 57]. Alignment shows incorporation of different exons at the 5' and 3' ends [63]. Responsiveness of *ZnT5* to zinc has produced conflicting results [57, 64]. *ZnT5* may represent a mode of regulation for a

ZnT gene that involves gene repression and mRNA stabilization in dietary and cell culture studies [63]. A suggestion that ZnT5 participates in zinc influx and efflux [65] is a unique concept that has yet to be further explored.

2.6. *ZnT6 (SLC30A6)*

ZnT6 was identified through searches of Expressed Sequence Tag (EST) database with homology to the amino acid sequences of mZnT4 [61]. ZnT6 has six TMDs and the histidine rich loop found in most ZnT proteins. In yeast, ZnT6 overexpression produces growth inhibition when cytoplasmic zinc is low. Cells are rescued when the cytoplasmic zinc level is high. A function in transport of cytoplasmic zinc into the TGN and vesicles has been proposed for ZnT6 [56, 61]. These proposals are consistent with known functions of ZnTs and the ZnT5/ZnT6 interaction needed to activate TNAP [59, 60].

mZnT6 mRNA was found in liver, brain, kidney, and small intestine. The protein was found only in brain and lung, suggesting post-transcriptional regulation for tissue-specific expression of ZnT6 protein [61]. Other immunohistological studies found ZnT6 protein was detected in chief cells of the stomach and epithelium of the jejunum, cecum, colon, and rectum [58]. Proposed roles for ZnT6 at these locations were cytoplasmic zinc transport into functional secretory granules. These include HCl and pepsinogen secretion for the stomach and zinc excretion via exocytosis in the lower gastro-intestinal tract [58] (see chapter 23 for further details).

2.7. *ZnT7 (SLC30A7)*

ZnT7 was identified from EST databases through homology to ZnT1 [66]. ZnT7 has the six TMD and histidine loop signatures of ZnT proteins. ZnT7 transcripts are detected in murine kidney, spleen, heart, brain, and lung with abundant expression in small intestine and liver [66]. ZnT7 protein was detected only in lung and epithelium of the small intestine [58, 66]. Transfection of Chinese hamster ovary cells (CHO) for ZnT7 overexpression leads to zinc accumulation in the TGN. This localization is proposed to be vesicular and different from that observed for ZnT2-ZnT6 in a variety of lines of cultured cells. Disruption of ZnT7 produces minimal changes in TNAP activity.

ZnT7 knockout mice display a zinc-deficient phenotype based upon reduced zinc content of the serum, liver, bone, kidney, and small intestine [67]. The *ZnT7^{-/-}* mice also have reduced food intake and body fat, but do not have the dermatological signs of zinc deficiency. Supplemental dietary zinc at six times the requirement for rodents did not correct the deficiency. ZnT7 appears to be highly expressed in adipose tissue and may relate to the low body fat of the *ZnT7^{-/-}* mice [67]. Consistent with defective responsiveness to dietary zinc is that *ZnT7^{-/-}* mice exhibit a reduced ability to accumulate orally administered ⁶⁵Zn into all tissues examined. ZnT7 protein is expressed throughout the small and large intestine [58] thus corresponding to regions of zinc absorption. This localization of ZnT7 and the reduction in zinc absorption in *ZnT7^{-/-}* mice [67] suggests ZnT7 could have a role in transcellular zinc movement through polarized epithelial cells. This could explain in part a vectoral transcellular transport of zinc within ZnT7 containing vesicles [68].

2.8. ZnT8 (*SLC30A8*)

β-cells of pancreatic islets accumulate very high amounts of zinc. Insulin is believed to be stored within secretory vesicles of β-cells as a hexamer bound with two zinc ions [69]. Since the availability of radioactive zinc in the early 1940's the pancreas has been recognized as an organ of high zinc turnover [reviewed in 22]. Zinc in β-cells has been imaged with fluorogenic probes [70, 71]. A relationship between zinc and insulin secretion/glucose homeostasis has a 40+ year literature base [reviewed in 72]. A direct relationship was established with the linkage of ZnT8 to glucose-induced insulin secretion [73, 74]. ZnT8 is localized to the membrane of the insulin storage granule [75]. ZnT8 uses the proton gradient produced by an ATPase to exchange two protons for the divalent cation Zn²⁺ in the granule. There Zn²⁺ forms complexes via histidine residues of insulin peptide.

ZnT8^{-/-} mice were produced, but no phenotypic changes compared to wild-type mice were identified in gross anatomical or behavioral characteristics or in body weight [76]. Zinc content of pancreatic islets were reduced in the null mutants with glucose-stimulated insulin secretion from isolated islets reduced by ~33% compared to wild-type controls. Blood glucose levels were not influenced by the null mutation, but plasma insulin concentrations showed a gender-specific reduction of 31-47% [76]. A simultaneous publication reporting phenotypic changes in another strain of *ZnT8^{-/-}* mice showed comparable results [77]. However, feeding a high-fat diet to the null mutants produced glucose intolerance with islets becoming less responsive to glucose. All data from the ZnT8 knockouts are consistent with this transporter having a role in insulin packaging in β-cells and impaired insulin secretion.

Considerable epidemiologic evidence with humans supports a relationship between zinc, diabetes, and ZnT8. Some of these studies have used the rationale that zinc has a role as an antioxidant and/or influences insulin secretion. For example, higher zinc intake in a large population study of women was associated with a slightly lower risk of type 2 diabetes [78]. ZnT8 was identified as a novel risk locus for type 2 diabetes in multiple studies [79-82] after the diabetes/ZnT8 link was established [73]. Subsequently, ZnT8 single-nucleotide polymorphisms (SNPs) were associated with reduced insulin secretion after stimulation with glucose but not insulin resistance [83]. In non-diabetic subjects with a family history of the disease, a SNP at amino acid 325 was associated with an increased circulating proinsulin-to-insulin ratio and decreased insulin responses following intravenous glucose tolerance tests [84, 85]. ZnT8 has been suggested as a factor in the pro-inflammatory origins of diabetes via β-cell apoptosis [86]. ZnT8 was identified as a major autoantigen for type 1 diabetes [87, 88]. A common polymorphism in hZnT8 at amino acid 325 (R325W) is a key determinant of two of the three major conformational epitopes of the protein [89]. The humoral type 1 diabetes autoimmunity to ZnT8 is directed against self not non-self epitope determinants [87]. Mutagenesis of mZnT8 to arginine (as in human ZnT8) allowed for reactivity with human autoimmune sera [89]. The R325W allele appears to encode a ZnT8 with less transport activity [90]. For further details of zinc and diabetes see chapter 25.

2.9. ZnT9 (*SLC30A9*)

ZnT9 has not received much attention. The protein may have six TMDs, has a putative cation efflux motif, a DNA excision repair motif and a nuclear receptor interaction

sequence [91]. It associates with cytosol and nuclear fractions, but not membranes. The function of the protein, considering its known characteristics, could be unique.

2.10. ZnT10 (*SLC30A10*)

ZnT10 has also received limited attention. Using homology to *ZnT1*, the *ZnT10* sequence was identified. Its expression appears, from limited data, to be restricted to fetal development [13].

3. ZIP (SLC39) Family

ZIP (solute-linked carrier 39) proteins are responsible for increasing intracellular zinc concentrations either through zinc import into cells or export from vesicles or organelles [10, 92] (Figure 1). The term for mammalian ZIPs originated from homologs defined in lower organisms as Zrt-, Irt- like proteins [92]. Most ZIP proteins have eight TMDs with N- and C- termini having an extracellular location. For some ZIP proteins the zinc channel is predicted to be TMDs IV and V [93]. Phylogenetic relationships among the homologues of the ZIP family are shown in Figure 4.

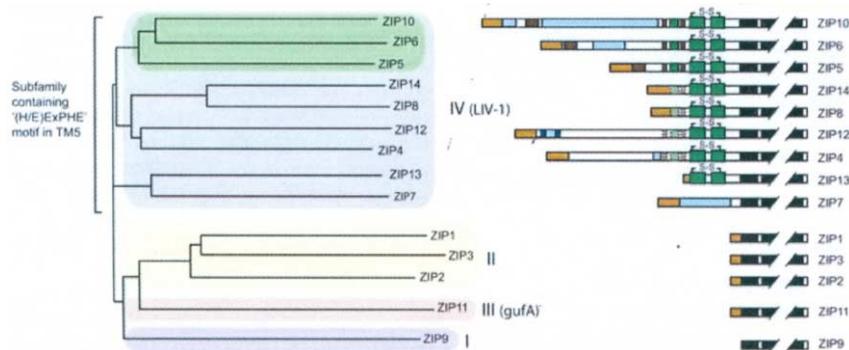


Figure 4. Dendrogram showing the phylogenetic relationships among the fourteen homologs of the ZIP family and the molecular organization of N-terminal extracellular domains of the ZIP proteins. From G. Schmitt-Ulms et al. [94].

3.1 ZIP1 (*SLC39A1*)

Mammalian ZIP1 was identified through homology with a Zrt-, Irt protein from *Anabidopsis thaliana* [92]. ZIP1 is expressed in many tissues and cell types [95, 96]. hZip1-mediated zinc transport in K562 cells are saturable and energy-independent with ZIP1 located at the plasma membrane [95]. Antisense RNA directed against hZip1 abolished zinc uptake indicating ZIP1 is essential for zinc transport in these erythroid-like cells [95]. In some cell types, ZIP1 is localized to the endoplasmic reticulum [97]. Treatment of human prostate cancer cells with prolactin or testosterone increases ^{65}Zn uptake and Zip1 mRNA levels [36]. This was the first demonstration of a Zip gene responding to hormonal stimulation. Zip1 mRNA levels were responsive to zinc supplementation in whole blood RNA from humans [21], but not in cultured THP-1 cells [98].

Zip1 mRNA is detected in all tissues of the mouse with the exception of the pancreas [22, 99]. The transcript levels in intestine and visceral endoderm do not respond to changes in dietary zinc, i.e. tissues involved in nutrient absorption [99]. ZIP1 is found localized to organelles in cells cultured in zinc adequate medium. Zinc restriction caused ZIP1 recruitment to the plasma membrane [100]. A di-leucine sorting signal is essential for endocytosis of ZIP1 [101]. Knockout of *Zip1* produces no difference in phenotype with adequate zinc in the diet. Embryo survival is diminished in the *Zip1*^{-/-} dams with zinc restriction [102].

3.2 ZIP2 (*SLC39A2*)

Human Zip2 was identified by homology to zinc transporters in fungi and plants [103]. Murine Zip2 was identified through similarity with hZip2 [99]. Expression of hZip2 is low and appears limited to the prostate, uterus, cervical epithelium [reviewed in 11], as well as monocytes and optic nerve [104]. Both hZip2 and mZip2 transfection into cells increases zinc uptake ability [99, 103]. Zip2 mRNA levels do not respond to dietary zinc restriction of mice [23, 99]. In contrast, treatment of THP-1 mononuclear cells or human PBMCs with TPEN, a cell-permeable metal chelator with high specificity for zinc, markedly up-regulates Zip2 mRNA levels [98, 104]. This suggests mononuclear cell *Zip2* may have an atypical mode of regulation. *Zip2*^{-/-} mice exhibit no overt phenotypic differences, but are more sensitive to dietary zinc deficiency during pregnancy [105].

3.3 ZIP3 (*SLC39A3*)

hZip3 was identified from comparisons with Zip genes in fungi and plant using mammalian ESTs [103]. mZIP3 and hZIP3 have very high protein homology. Zip3 expression is low in most tissues [22, 99] except the testes [99]. Zip3 does not appear to be regulated by dietary zinc [22, 99]. Cell transfection studies demonstrate that ZIP3 can transport zinc, but other metals inhibit this process suggesting multiple metal substrate affinities [99]. ZIP3 localizes to organelles unless they are placed in low zinc medium [100].

Zip3 null mutant mice display normal growth and have minimal effects upon dietary zinc restriction [102]. In cultured mammary cells however, Zip3 was found to be prolactin-regulated and is required for zinc transport into these cells [106]. Double knockouts of Zip1 and Zip3 and triple knockouts of Zip1, Zip2, and Zip3, were of normal phenotype unless a zinc deficient diet was fed [102, 107].

3.4 ZIP4 (*SLC39A4*)

The acrodermatitis enteropathica (AE) syndrome was identified as a zinc absorption defect more than three decades ago [108]. This zinc-malabsorption disorder is corrected by supplemental zinc. AE is a rare, autosomal, recessively inherited disease and mapped to human chromosome 8 [109]. Genetic screening of the AE gene region demonstrated a gene of the Zip family that was designated Zip4 [110]. The gene was shown to be highly expressed in human small intestine, stomach, colon, and kidney [110]. Multiple mutations in *hZip4*, including missense mutations, splicing defects and transcription-inactivating upstream deletions have been identified in AE patients [110-112]. Some mutations may produce ZIP4 retention in the endoplasmic reticulum [113].

The skin lesions and immune defects of AE can be overcome through consumption of an oral zinc supplement [108]. Neurological defects of AE may not be corrected. The effectiveness of the zinc therapy suggests AE produces a ZIP4 protein of diminished transport efficiency/defective plasma membrane localization that can be compensated by high intakes of zinc and/or other metal transporters in the gastrointestinal tract. A comparable intestinal zinc transport defect, Lethal Trait A46, has been observed in the Holstein breed of cattle and is likely attributed to defective bovine *Zip4* expression [114].

Mouse and human ZIP4 proteins are conserved and share 76% homology [112]. During zinc deficiency in mice ZIP4 localizes to the apical plasma membrane of enterocytes [22, 113] and embryonic yolk sac [113]. This increase in apical orientation with zinc restriction corresponds to the increased intestinal zinc uptake observed in those conditions [7, 8]. *Zip4* expression appears to be regulated by both transcriptional and post-transcriptional mechanisms. Transcriptional regulation in response to dietary zinc is in part via the transcription factor, Krüpple-like factor 4 (KLF4). KLF4 is induced by dietary zinc restriction of mice [115] and cellular zinc chelation in intestinal/colonic epithelial cells [115-117]. KLF4 is highly expressed in the gastrointestinal tract [118]. Zinc repletion was reported to cause *Zip4* mRNA degradation and rapid ZIP4 endocytosis [119]. The histidine-rich intracellular domain may have a role in ZIP4 endocytosis, ubiquitination and degradation [120]. Dietary zinc restriction causes a removal of the extracellular amino-terminal ectodomain of ZIP4 which suggests proteolytic cleavage is a control point for ZIP4 regulation [120, 121].

Homozygous knockout of *Zip4* produces embryonic lethality [122]. This outcome is preventable through excess zinc given to the dams orally or intraperitoneally. Heterozygous (*Zip4*^{+/−}) mice are more sensitive to zinc restriction and show growth retardation and morphologic abnormalities [122].

Microarray profiling identified *Zip4* mRNA as markedly up-regulated in human pancreatic cancer [123]. ZIP4 protein overexpression and higher *Zip4* mRNA were confirmed in individual tumor samples [124]. ZIP4 overexpression in MIA PaCa-2 human pancreatic cells increased zinc accumulation and cell proliferation. In nude mice, ZIP4 overexpression through MIA-ZIP4 cell inoculations increased tumor weight and ascites incidence as well as higher zinc content and greater proliferation [124]. ZIP4 down-regulation with siRNA inhibited tumor growth and increased survival in mice with pancreatic xenographs [125]. It was proposed that ZIP4 via zinc transport activity influences CREB, a transcription factor involved in the control of IL-6/STAT3 pathway and numerous other factors related to proliferation [125-127]. Increased ZIP4 expression was also found in hepatocellular carcinomas. Evidence suggests ZIP4 suppresses apoptosis and increases *in vitro* migration [128].

3.5. ZIP5 (*SLC39A5*)

ZIP4 and ZIP5 proteins share 30% homology with mZIP5 and hZIP5 sharing 84% identity [113]. *Zip5* has high expression in liver, kidney, pancreas, small intestine and colon [113]. With an adequate supply of zinc ZIP5 is located at the basolateral membrane, but is internalized upon zinc restriction. *Zip5* mRNA is not influenced by zinc levels. It remains associated with polysomes during zinc deficiency and upon zinc repletion is mobilized for rapid synthesis [129]. ZIP5 transports zinc as a specific substrate in transfected cells [130]. The basolateral orientation of ZIP5 has led to the

proposal that it acts as mechanism for sensing zinc in enterocytes and pancreatic acinar cells [130].

3.6. ZIP6 (*SLC39A6*)

ZIP6 also called LIV-1 in early work was identified as a novel estrogen inducible-gene in breast cancer cells [131]. ZIP6 is one of nine members of the LVT subfamily of ZIP transporters. They contain the signatures of ZIP proteins, but also have a unique, conserved, putative metallo-protease motif (HEXPHEXGD) at TMD V [132, 133]. Cell transfection studies demonstrate ZIP6 is a zinc transporter, localized to the plasma membrane [133]. Zip6 is expressed in numerous cancer cell lines e.g. HeLa, lung carcinomas as well as metastatic breast cancer cells suggesting ZIP6 has a role in cancer progression [133, 134].

The responsiveness of Zip6 to estrogen has led to identification of ZIP6 as a reliable marker for estrogen-receptor positive cancers [135, 136] and to detect luminal A type breast cancer [reviewed in 10, 137]. At the mechanistic level STAT3 was shown to activate *Zip6*, which via zinc ion import, leads to translocation of the zinc-finger transcription factor snail to the nucleus to down-regulate genes that influence the epithelial-to-mesenchymal transition including E-cadherin [138]. Similar to this evidence with a zebrafish model came from the Zip6 homologue in *Drosophila*, termed *fear of intimacy*, which has a comparable effect on DE-cadherin needed for proper gonad morphogenesis [139].

ZIP6 transporter activity has been shown to regulate CD11c⁺ dendritic cells [140]. LPS down-regulated *Zip6* which caused increased surface expression of MHC class II molecules required for cell maturation. Zinc chelation with TPEN, a cell permeable chelator, produced the same effect as Zip6 down-regulation. Supplemental zinc had the opposite effect. This suggests that the transported zinc via ZIP6 activity served a regulatory in immune cell activation (see chapters 10 and 13).

3.7. ZIP7 (*SLC39A7*)

Zip7 was identified through homology to the *mKE4* and *hKE4* which map to the major histocompatibility complex of respective chromosomes [141]. Subsequent alignment comparisons identified them as *Zip* family members [142]. ZIP7 is a LIV1 subfamily member. It is ubiquitously expressed in tissues and is of high abundance in breast cancer [133]. Transfection of *Zip7* into cells increases intracellular zinc based upon responses of a fluorescent probe. ZIP7 localizes to the TGN and endoplasmic reticulum [133, 143]. *Zip7* and ZIP7 protein do not appear to be regulated by zinc status.

An important aspect of ZIP7 function is its relationship to breast cancer [144]. Studies with breast cancer cell lines showed that ZIP7 expression is required for increasing intracellular zinc levels. These levels correspond to activation of EGFR, Src and IGF-1R signaling molecules as well as increases in growth and invasive behavior. Recently it has been proposed that ZIP7 mediates release of zinc from stores in the endoplasmic reticulum and may be required for tyrosine kinase activation [145]. This may occur through phosphatase activity inhibition produced by increases in labile zinc concentrations. A mechanism for zinc transporter-mediated phosphatase inhibition has been proposed for ZIP8 [146].

3.8. ZIP8 (*SLC39A8*)

Zip8 was originally named BIGM103 as the gene was induced in primary human monocytes after exposure to a *Bacillus* cell wall antigen [147]. Zip8 expression is found in lung, kidney, testes, liver, brain, small intestine, T-lymphocytes, and the membranes of mature RBCs (red blood cells) [21, 24, 147-149]. Zip8 expression appears to be refractory to dietary zinc status, but is highly influenced by immune mediators. MDCK cells transfected with *Zip8* show a plasma membrane localization of this protein during zinc restricted conditions, but is internalized with adequate zinc [148]. ZIP8 abundance at the RBC membrane of mice is unaffected by dietary zinc deficiency [24]. In contrast, ZIP8 in human T-cells is localized to the lysosomal membrane with colocalization with lysosome-specific markers, i.e. LAMP1 and Lysotracker [146]. The reason for these differences is not known. ZIP8 has been demonstrated to act as a cation (Cd, Mn, Zn) transporter in the *Xenopus* oocyte system [150, 151] and in mouse fibroblast cultures [151, 152]. In the *Xenopus* model Cd²⁺ and Zn²⁺ transport is bicarbonate (HCO₃⁻) coupled [151]. The natural substrate(s) for ZIP8 transport is not known however zinc concentrations are higher in the peripheral circulation than are those for cadmium (a non-essential metal) or manganese (an essential metal).

Since the inducibility of Zip8 by immune-mediators was identified including LPS and tumor necrosis factor (TNF)- α in monocytes [147], newer studies focused on T-cells and the lung. For example, TNF- α stimulated Zip8 expression in primary human lung epithelia and in human upper airway epithelial cells [149]. TNF- α induced a glycosylated ZIP8 that localized to the plasma membrane and mitochondria. A concomittent increase in cellular zinc was coincident with an increase in cell survival following TNF- α . Inhibition of Zip8 expression with siRNA reversed the cellular zinc content and decreased survival. At the same time, other studies of Zip8 focused on T-cells. Previous work had shown that human T-cells obtained from donors were high expressers of Zip8 [21]. Zinc supplementation of those subjects did not increase Zip8 expression, but it did augment the production of interferon (IFN)- γ in T-cells upon activation. Of the transporters in T-cells that responded to immune activation only Zip3, Zip8, and Zip14 produced a significant response. The 14-fold increase in Zip8 being the greatest response [146]. Zinc added to T-cells in vitro was found to increase IFN- γ and perforin expression and secretion, both signatures of activation with a maximum effect at 3.1 μ M. Knockdown of Zip8 by siRNA decreased ZIP8 levels and reduced secretion of IFN- γ and perforin. Overexpression of Zip8 had the reverse effect showing enhanced activation. Confocal microscopy showed ZIP8 localized to the lysosome where abundance was increased upon activation. Loss of lysosomal labile zinc, measured with a zinc fluorophore, coincided with activation. Zinc at concentrations at 3.1 μ M reduced calcineurin (CN) phosphatase activity as did both a CN inhibitor and Zip8 overexpression. The results suggest a model, shown in Figure 5, where Zn²⁺ transported by ZIP8 from lysosomal stores, inhibits CN phosphatase activity, sustains phosphorylation of the transcription factor CREB (the active form) and hence increases transcription of the *IFN- γ* gene. This event may be an example of the fine control zinc provides in cell signaling.

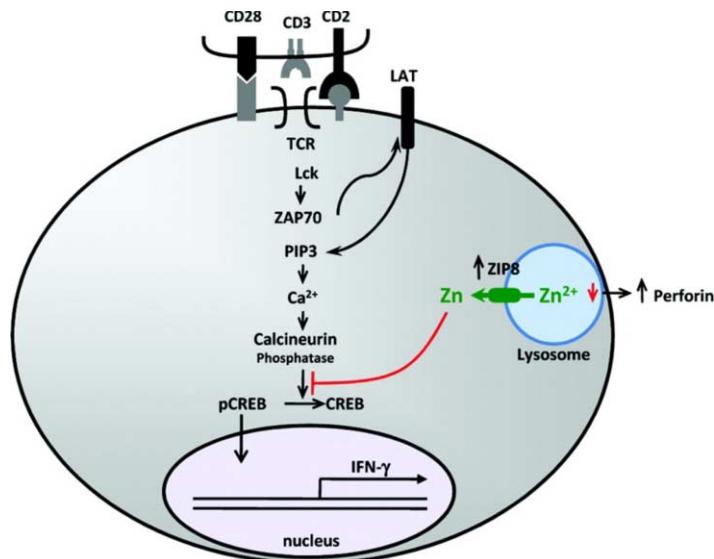


Figure 5. Proposed model for the signal transduction pathway connecting antigen activation of the T-cell receptor where Zn^{2+} ions transported by ZIP8 inhibits calcineurin phosphatase activity to increase IFN- γ production. From T. Aydemir et al. [146] Reproduced with permission of the J. Leukocyte Biology.

Other roles of ZIP8 have not been examined. An internet-based search has found information indicating that a homozygous *Zip8* null mutation leads to embryonic lethality. This suggests ZIP8 performs a critical function during development.

3.9. ZIP9 (*SLC39A9*)

Sequence homology of both human and mouse ZIP9 places it in the ZIP family. It is the only member of the subfamily I of mammalian ZIP transporters [94, 132]. No descriptions of structure, localization, function or regulation on ZIP9 have appeared.

3.10. ZIP10 (*SLC39A10*)

ZIP10 is in the LIV-1 subfamily of ZIP proteins [94, 132]. Its closest homology is to ZIP6 and ZIP5. *Zip10* was identified as upregulated as a serendipitous outcome through evaluating the genotypic effects of a liver-specific MTF-1 conditional knockout mouse model [153]. Metal-response element-binding transcription factor-1 (MTF-1) is a zinc finger protein that upon binding Zn^{2+} in the cytosol translocates to the nucleus where it binds to a “metal-response element” (MRE) [154, 155]. This activity frequently produces zinc-responsive genes. MTF-1 is known to regulate two zinc transporters ZnT1 and ZnT2 [17, 33]. These zinc regulated genes have one or multiple copies of the MRE consensus sequence (TGCRNC). Homozygous MTF-1 disruption is embryonically lethal [156].

ZIP10 is widely expressed in mouse tissues with high abundance in brain, mature RBCs, liver, small intestine and kidney [reviewed in 10]. A ZIP10 identified in rat renal brush border [157] was upon further analysis shown to align more closely with hZip4 [158] and has been assigned a Zip4-like designation. Zip10 in zebrafish is highly

expressed in gill and kidney [158]. Regulation of *Zip10* reflects the influence exerted by MTF1. Specifically, availability of zinc represses *Zip10* expression in both zebrafish and mice [158, 159]. In contrast, *Zip10* upregulates upon zinc restriction of cells or intact mice [159]. The MRE of *mZip10* starts at +17 downstream of the transcription start site. This location restricts Pol II movement upon MTF-1 binding; a condition associated with available zinc. In contrast, in zinc restriction MTF-1 remains in the cytoplasm and *Zip10* transcription is not repressed.

Screening of breast cancer samples established that *Zip10* was significantly associated with metastasis to the lymph node [160]. This finding further supports the relationship of the LIV1 proteins with breast cancer [132, 137]. *Zip10* mRNA levels were higher in invasive and metastatic breast cancer cell lines. Migratory activity of these cells was inhibited by ZIP10 knockdown with a corresponding reduction in intracellular zinc. Such findings suggest ZIP10 as well as ZIP6 are both useful markers for the metastatic breast cancer phenotype and form novel drug design targets (see chapter 14).

Analysis of the evolutionary descent of the ZIP family has led to recognition of the homology of ZIP10 and its paralogs ZIP5 and ZIP6 with the prion protein (PrP)[94]. When cellular PrP, which is expressed at high levels, undergoes a structural transition to the disease-causing PrP^{sc} form, neurologic disease is produced. These diseases are neurodegenerative and include Creutzfeldt-Jakob disease in humans, scrapie in sheep and spongiform encephalopathy in cattle. Homology of PrP with ZIP10 is at the N-terminus which for both is an extracellular domain [94]. The concurrent high expression of both ZIP10 and PrP in the central nervous system is of interest but currently a relationship is limited by proximity as they can be purified together. Of particular note is that it has been proposed earlier that PrP could be a zinc transporter or zinc sensor [161]. Metal binding to PrP has been shown to stimulate its endocytosis [162].

3.11. ZIP11 (*SLC39A11*)

ZIP11 is the only member of the gufA subfamily of ZIP transporters. No descriptions of structure, localization, function or regulation for ZIP11 have appeared.

3.12. ZIP12 (*SLC39A12*)

ZIP12 was identified in a genetic screening of a schizophrenia susceptibility locus on chromosome 10p in order to detect proteins potentially involved in zinc transport. Brains of schizophrenics have a lower zinc concentration than brains of individuals without the disorder [163]. A missense homozygous mutation in *Zip12* and frequency of schizophrenia was detected in a small study [164]. ZIP12 is in the LIV-1 subfamily with *Zip4* being its closest paralog [94]. Localization to the brain is likely, but has not been demonstrated. No description of structure, function or regulation for ZIP12 has appeared.

3.13. ZIP13 (*SLC39A13*)

ZIP13 is a member of the LIV-1 subfamily of ZIP transporters [94, 132]. It is closest in homology to the paralog ZIP7. A detailed study of Ehlers-Danlos syndrome (EDS), a human disorder characterized by progressive kyphoscoliosis, joint hypermobility and

hyperelasticity of skin and severe hypotonia of skeletal muscles [165]. Genomic and cDNA sequencing of EDS patient DNA established these individuals were homozygous for a 9bp in-frame deletion of exon 4 of *SLC39A13*. The homology to ZIP7 and its localization to the TGN let to the suggestion that ZIP13 was intracellular [165].

The identification of the EDS/ZIP13 relationship was rapidly followed by production of a homozygous *Zip13* knockout mouse [166]. *Zip13*^{-/-} mice have reduced osteogenesis, abnormal cartilage development, reduced dentin and alveolar bone, abnormal craniofacial and decreased dermal and corneal stromal collagen. ZIP13 was established to be localized to the Golgi in wild type mice. The *Zip13* knockout caused dysregulation of BMP/TGF- β (bone morphogenetic protein, BMP; transforming growth factor beta, TGF- β) mediated gene expression including those that are essential for bone, tooth, and craniofacial development. BMP and TGF- β receptor activation leads to SMAD protein translocation to the nucleus for their role as transcription factors (see chapter 13). Many SMAD (intracellular transcription factors for TGF- β signaling) proteins have a zinc-binding motif in their DNA binding domain [167]. ZIP13 transporter could influence zinc availability for that motif. Alternatively, Smad proteins are phosphorylated downstream of BMP or TGF- β rector complex activation. Phosphorylation is necessary for translocation to the nucleus to initiate transcription. Inhibition of phosphatase activity, as demonstrated for a mode of ZIP8 action via zinc transport [146] could in this situation also retain phosphorylation, and thus sustain nuclear translocation. These ZIP13-directed effects could be a factor in regulating events that are defective in EDS. It is not known if *Zip13* is zinc regulated, but some features of human zinc deficiency [7, 8] e.g. delayed growth, are similar, to those of EDS.

3.14. ZIP14 (*SLC39A14*)

ZIP14 is a LIV-1 subfamily member of ZIP proteins, with ZIP8 being its closest paralog [94, 132]. The amino acid sequence in TMD V that unites other LIV1 proteins is slightly altered in ZIP14 [132]. Zinc uptake experiments in transfected cells demonstrated that ZIP14 transports zinc [168-172]. The proposed zinc transport channel is between TMDs 4 and 5 [169]. Of note is that *Zip14* was originally identified among a group of genes expressed during adipocyte differentiation [173, 174]. *Zip14* expression correlates with adipogenesis [174]. ZIP14 is expressed in many tissues with small intestine, liver, pancreas, heart, brain, kidney, testes, and T-cells having high expression levels based on array data transcript abundance and western analysis [146, 169-172]. Localization appears to be primarily to the plasma membrane [169-171], including transfected cells. In transfected Madin-Darby canine kidney cells (MDCK), which are polarized epithelial cells, ZIP14 is localized to the apical surface [172].

The transport capabilities of ZIP14 have been evaluated from different perspectives and with different systems. With *Zip14* transfected HEK293T cells, ZIP14 was at the plasma membrane and zinc transport was established by increased labile zinc, as measured by Fluozin-3AM fluorescence, ⁶⁵Zn incorporation and increased metallothionein mRNA as a reporter for increased MTF-1 activity from intracellular zinc binding [169]. Subsequently ZIP14 was also shown to transport non-transferrin-bound iron (NTBI) in the HEK 293T and Sf9 heterologous systems [170]. Evidence for transport was produced by ⁶⁵Zn and ⁵⁹Fe incorporation and *Zip14* knockdown with siRNA. Zinc was shown to be a potent inhibitor of NTBI. In the MDCK cell model and

using radionucleotide uptake as the measure, *Zip14* transfection stimulated the uptake of zinc, manganese and cadmium [172]. Zinc powerfully inhibits cadmium uptake. Cadmium uptake was dependent upon extracellular HCO₃. ZIP14 isoforms produced by the two (A and B) splice variants of Zip14 show different kinetics for cadmium uptake. These findings and others soon to be published show that ZIP14 is capable of transporting a number of metal ions, but that zinc is the likely physiological substrate. This does not rule out the possibility that during disorders of iron metabolism, ZIP14 may participate in NTBI transport [reviewed in 10]. Furthermore, ZIP14 may be capable of transporting multiple metal ions from endosomes as has been shown for transferrin bound iron [175].

mZip14 is located on chromosome 14. At least two distinct transcripts have been identified [169, 176]. The reference sequence containing the entire Zip14 coding sequence is Zip14A. The other sequence has a shorter 3' untranslated region. This splice variant is termed Zip14B. The Zip14A transcript contains exon 5 located at bps 710-879, whereas Zip14B contains exon 3 at 713-882. Both variants produce Zip14 proteins that transport metals, but exhibit some differences in tissue distribution. Of note is that differences in the abundance ratio of Zip14A to Zip14B have been observed in tissues from colorectal cancer patients [176], suggesting these splice variants may have clinical and functional relevance.

Regulation of *Zip14* by immune mediators has attracted considerable attention. Zip14 mRNA was identified (as KIAA 0062) by differential display analysis as differentially expressed in primary human fibroblasts as induced by interferon α [177]. Subsequently Zip14 mRNA was identified as the most highly responsive transcript of a screen of ZnT and Zip transporter mRNAs from liver in response to two proinflammatory murine models, i.e. sterile abcess (turpentine administration) and LPS [169]. Hypoferremia and hypozincemia are among the classical changes observed across species during the acute-phase response [178]. The purpose of these changes are not known, but speculation focuses on reduction of iron and zinc availability for pathogenic microorganisms, or in the case of zinc, make the micro-nutrient available for cellular functions such as regulation of signaling pathways. The latter is supported by the finding that Zip14 mRNA is stimulated significantly in primary human T-cells upon TLR-mediated activation [146]. In response to cytokine treatment and inflammation, zinc is redistributed to many tissues, particularly the liver [179]. The turpentine inflammation model produces increases in IL-6 and leptin mediated by IL-1 β . IL-6 is the major proinflammatory cytokine regulator for acute phase gene activation [178, 180]. The induction of Zip14 by turpentine inflammation in wild type mice with no response in IL6^{-/-} mice, established *Zip14* as an acute phase responding gene [169]. *Zip14* has also been shown to be induced by IL1 β via a mechanism that is dependent upon nitric oxide (NO) via stimulation of inducible nitric oxide synthase [171]. NO interacts with the *Zip14* promoter to increase ZIP14 synthesis with transfer to the plasma membrane for enhanced zinc transport. Roles of ZIP14 in non-hepatic tissues have yet to receive much attention.

4. Conclusion and Perspectives

Inquiries about the physiological relevance of the zinc transporters usually include two major questions:

- Why are there so many zinc transporters compared to those needed to regulate copper and iron metabolism/function?
- What does the transported zinc do?

Clearly the answers to these questions relate to the structural, catalytic and regulatory functions of zinc [7, 8]. Also needed to be factored into this perspective is an appreciation that zinc is a stronger Lewis acid (electron acceptor) than iron (Fe^{3+}) but weaker than copper (Cu^{2+}). This favors strong binding to thiolate and amino electron donors [2]. Fast ligand exchange is a factor in zinc function. Zinc is not a redox metal, but through zinc-thiolate clusters, oxidants are reduced and produce zinc release and disulfide bond formation [181]. Another potential to explain why there are so many zinc transporters may rest with the comparative functions of zinc compared to iron and copper. Zinc has multiple functions in mammalian systems based on a plethora of evidence. In contrast, iron functions for oxygen transport and for iron metalloenzyme activity. Copper is principally used for copper metalloenzyme production and activity. Hence the multiple functions of zinc plus the wide spread cellular location for those functions may require multiple transporters to satisfy these requirements.

Among things to be considered for future research include the following:

- Interactions between zinc transporters, effects of zinc on transporters for other substrates [27, 28, 94, 182], interaction of proteins and other molecules with N-terminal domains of zinc transporters .
- The evolving understanding of zinc on regulation of cell signaling pathways via zinc transporter activity [183-186]
- A forty year knowledge that zinc inhibits gastric-acid secretion and acts as an emetic but no understanding of how this process is regulated [187, 188]
- A role for zinc transport in the as yet unexplained effects of zinc on carbohydrate metabolism [189, 190]
- Which transporters are likely to be targets for drug development [73, 90, 116, 145]
- Use of both ZnTs and ZIPs as markers for diseases.
- The demonstration that zinc actively influences enzyme activity through transport activity in vivo as can be shown in vitro, particularly the phosphatases [62, 146, 191].
- Detailed of mechanisms for zinc transport to establish if, as with transport in microorganisms [192], that these proteins serve as selective electrodifusional channels facilitating passive movement driven by zinc concentration gradients.

These are among the research questions that will help to address what transported zinc does and why multiple zinc transporters have evolved. These topics all fall within the notion that zinc participates produces in quasi-hormonal effect [2].

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9. Measurement and Imaging of Free and Total Zinc in Biological Specimens

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Abstract: Numerous methods have proven useful in the study of zinc in biological systems. This chapter briefly reviews several methods for determining and imaging zinc, particularly in aqueous solutions, cells, and tissues. The sections are written for bioscientists who are potentially interested in a method but may have little experience with it. The methods include fluorescent stains for zinc, fluorescence-based biosensors, X-ray fluorescence, determination of zinc binding affinity, and fiber optic biosensors. For each method the principle and background are described, followed by a brief summary of the procedure and typical results, and concluding with the advantages and disadvantages of each method.

Keywords. Zinc, fluorescence, colorimetry, binding, sensitivity, microscopy

Introduction

Measurement of zinc ions in biological specimens is essential to understanding zinc biology. Understanding the biological roles or effects of zinc becomes very difficult without knowing the amounts that are present, and usually what proportion is available for interaction with the biological molecule(s) of interest. Following a large effort (which is ongoing) there are a number of tools now available to analyze various biological specimens (cells, tissues, organs, and whole organisms, as well as fluids including serum, cerebrospinal fluid, growth media, sea water, and fresh water) for their zinc content. This chapter provides an overview of a number of these tools. Several of them are used in an imaging mode, due to the richness of information which may be obtained when zinc levels are related to biological structure. The brevity of this chapter makes it necessary to only provide a brief summary of the principles and important features of each technique; complete details may be found in the references in each subsection.

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1. Figures of Merit

Each of the methods described herein has a number of performance characteristics which define its utility to answer various research questions in zinc biology. These figures of merit make some techniques well suited to answer a particular question, and others totally incapable: for instance, X-ray fluorescence permits mapping of total zinc ion levels within a cell, but prolonged X-ray exposure of the specimen makes *in vivo* studies problematic. Among the important figures of merit for each method are **selectivity**, the degree to which the method also responds to other species, such as divalent cations; **sensitivity**, which is conveniently expressed as a **detection limit**, the minimum level of zinc which can be detected at some specified level of reliability; **dynamic range**, the range of zinc levels which can be quantified with some level of accuracy; **accuracy**, which is how close the measured value comes to the actual value; and **precision**, which is the average error in the measurement.

Other factors are also important in many determinations: reversibility of the response; destructiveness (or non-destructiveness) of the method; response time (time resolution) of the method, and whether data are reported in real time; applicability to imaging techniques; and specimen (sample) characteristics, including sample amount and live (vs. fixed or dehydrated) specimens. Other factors frequently of importance include cost and usability in particular places (e.g., outside the laboratory, or on board ship) or with particular specimens (e.g., live humans).

2. Organization of the Chapter

For each of methods, the background and physical principle of the method are briefly described, followed by a description of the procedure, typical results, and issues with the method, including advantages, disadvantages, and future prospects. The sections of this chapter are meant to be brief introductions to each method and its applications to questions of essentially biological interest: references in each section will guide the reader to more thorough treatments. The chapter is not a comprehensive listing of methods, and necessarily focuses on methods most useful to biologists; thus several important methods more useful for chemical or environmental analyses are not included. The methods included below are: identification of granular zinc in tissues with fluorescent staining; determination of free zinc in small samples by fluorescence intensity in the stoichiometric regime; colorimetric measurement of zinc binding affinity to proteins and other macromolecules; imaging of zinc in cells using small molecule fluorescent indicators; use of fiber optic sensors for measuring free zinc in remote or meso-volume specimens; quantitative microscopy of free zinc in cells by fluorescence excitation ratio using carbonic anhydrase-based indicators; and imaging total zinc and other metals by X-ray fluorescence.

3. Identification and Visualization of Zinc in Secretory Granules (Histochemically-Reactive Zinc)

3.1 Principle and Background

Some of the earliest data on zinc in biology come from research on the zinc that is secreted by exocrine glands. Delezene published extensively on the amounts of zinc

secreted into certain snake venoms and into the pancreatic juice of the exocrine pancreas[1]. Histochemical studies of zinc came later, with one sulphide study of a sea snail showing zinc in the hepatocytes (evolutionary precursors of granulocytes) in 1905 [2], and later dithizone studies[3] showing the rich deposits of zinc in both the exocrine and endocrine parts of the pancreas.

We now know that almost all zinc that can be labeled by histochemistry is selectively concentrated in the secretory granules of cells that secrete zinc. There are about a dozen cell types that do this, including a certain class of forebrain neuron that co-secretes zinc and glutamate [4], the endocrine and exocrine pancreatic secretory cells, the salivary gland (also as modified to a venom gland), the prostate gland, some (but not all) of the pituitary cell types, thyroid secretory cells, and all of the blood granulocytes as well as another presumed antimicrobial cell, the Paneth cell of the intestinal crypts.

Thus, whether one uses the venerable dithizone method, the silver methods of Timm as modified by Danscher, or the newer fluorescent methods [5] the pattern of staining is essentially the same. Zinc that is in the secretory granules of cells that secrete zinc stains abundantly, as does the secretory fluid generated by such cells wherever (for example in the secretory lumens of the prostate) the fluid can be stained and visualized histochemically. Beyond the secretory granules and secreted fluids, there is only scanty and scattered zinc staining anywhere in any normal tissue harvested while healthy. This selectivity presumably reflects the low affinity of the ligands with which the zinc is bound in the various secretory granules.

There are two major exceptions to this restricted localization of the staining, however. The first is any tissue that has been compromised. In fact, the appearance of zinc staining in cytosol, lysosomes, nuclei, or any other organelle is a sensitive indicator of dys-homeostasis in the cell [6]. This is seen prominently, for example, in neurons grown in culture, where many endosomes scattered through the cytoplasm stain for zinc. By contrast, neuronal tissue harvested from a healthy brain exhibits no such zinc staining in cytoplasmic endosomes. Importantly, neurons harvested from a brain that has been injured 2-24 hours previously (e.g., by ischemia and reperfusion) are filled with this “injury” biomarker of perikaryal zinc-filled endosomes. When grown in culture, cells of some classes that normally do not harbor zinc-filled endosomes also typically have them in abundance [7]. The second condition in which lysosomes, in particular, sequester abundant zinc is when the organism is exposed to high zinc concentration, as for example when zinc is added to the tank water of fish. Cells exposed to the water, such as gill cells, become loaded with zinc in that situation [8].

One final caveat concerning staining for zinc in tissue previously injured is important to mention. In the brain, injured neurons develop an affinity for derivatives of fluorescein. This is the basis of the well-known and widely used (if incompletely characterized [9]) family of fluorescent stains for injured neurons trade-named “Fluoro Jade”®. Fluoro Jades are mixtures of anionic fluorescein derivatives, and we have observed (Prough, Hawkins, Frederickson unpublished) that even ordinary fluorescein itself also selectively labels neurons injured 2-24 hours previously by brain trauma. Thus we would urge caution in the use of fluorescein-based indicators for zinc (or other analytes) in studying injured neurons, and suggest the use of non-fluorescein-based indicators (such as TSQ, 6-methoxy-8-quinolyl)-*p*-toluenesulfonamide) for this purpose, at least until the interaction between fluorescein dyes and injured neurons is better understood.

3.2 Procedure

Methods of preparing, embedding, cutting and counterstaining the Timm or Timm-Danscher material have been described in meticulous detail elsewhere [10], so we will focus on procedural points of particular interest in staining for zinc ion.

Fixing tissue masks all zinc staining unless (as is done for the silver stains) the zinc-binding moiety (sulfide or SeO_2^-) is introduced to the tissue before the fixative either by intravital intraperitoneal administration, by postmortem perfusion in situ, or by exposure to vapors after cryosectioning, but before vapor fixation.

For dithizone or fluorescence staining, tissue must be unfixed and freshly cut after thawing. Prior fixation masks the zinc staining in the granules, probably by the steric hindrance imposed by the cross-linked proteins. Tissue that has been cryosectioned and refrozen before staining will give poor results.

The best histologic picture is maintained when tissue is cut in the “Scandinavian” way, using a relatively warm chamber (-9 °C.), a relatively thick section (30 μm), and a “stretch plate” attached to the knife for catching and then sliding the tissue onto a glass slide to minimize trauma to the cut sections.

Tissue should not be bath-dipped into zinc stain as one would dip into a Nissl stain, for example. This is because some zinc will wash out into the bath, and immediately bind to the probe, then the zinctated probe molecules thus formed in the staining dish will adhere non-specifically to the tissue, introducing a random, artifactual background. Instead, one simply pipettes a fixed volume of the staining solution onto the section, waits, and pours off the excess. Rinsing after staining with isotonic fluid does not seem to improve specificity. We have never found a method of dehydrating and cover-slipping stained tissue that did not adversely affect the stain. Nor are we aware of any successful use of a counter stain before or after zinc staining. Instead, we use sequential sections with alternate staining, and water-immersion optics for higher magnifications.

3.3 Results

Perhaps the most vivid and “clean” staining of secretory granules is achieved by the fluorescent stain from the Lippard group, Zinpyr1 [11], although a lower-affinity lipophilic probe developed by Paul Bently promises higher specificity for the very highly-concentrated zinc in the vesicular pool [5]. In the hippocampus of the rat, for example, Zinpyr1 stains the entire mossy-fiber plexus (known as “stratum lucidum”) which is densely packed with large and giant axon terminals, as shown in the inset and left panel of figure 1. The smaller and sparser zinc-containing terminals in other strata also stain correspondingly lighter. Zinpyr is lipophilic and can penetrate through terminal cell walls as well as the vesicular membranes, so staining the intravesicular zinc is robust. Dyes that do not penetrate membranes, or that require enzymatic catalysis for fluorescence will not stain intravesicular zinc in fresh cryostat sections [11]. Zinpyr also stains injured and degenerating neurons vividly[11], but as mentioned above, the affinity of fluoresceins for some unknown ligands in degenerating neurons makes that staining untrustworthy as concerns the presence or location of zinc.

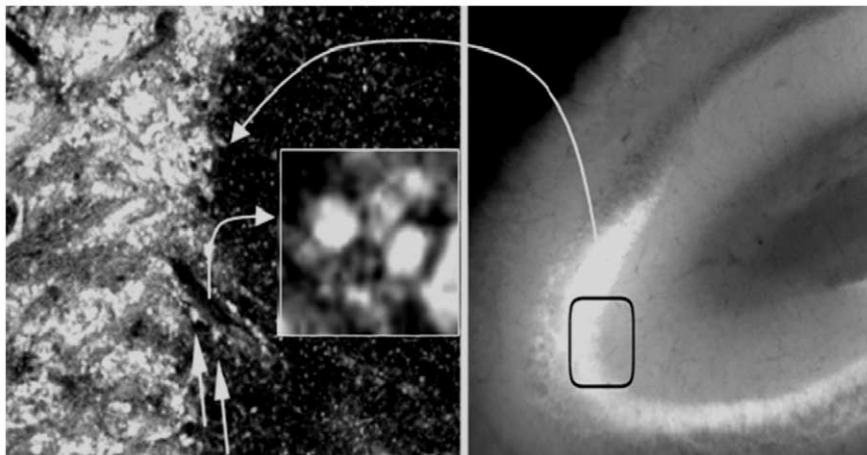


Figure 1. Zinpyr-1 stained fluorescing axon terminals in the hippocampal formation. The right panel is a low-magnification survey showing the brightly-fluorescent band of mossy-fiber axon terminals. The right inset is magnified in the left panel, showing the stark contrast between the mossy fibers, densely-packed with large and giant terminals, and the adjacent dark stratum, where zinc-containing terminals are sparse. Inset in the left panel shows two giant terminals, about 10–15 μm in diameter.

3.4 Issues

For determining the position and morphology of the zinc granules in cells and tissue sections, the fluorescent methods are quick and easy. Moreover, inasmuch as they bind zinc with fixed stoichiometry, they give reasonably reliable ordinal scale quantification of the amount of zinc in secretory granules [12]. The silver methods of Danscher [10], on the other hand, are far superior for high magnification light or for electron microscopy. They can also be counterstained and, unlike the fluorescent material, the silver-stained slides and grids can be kept indefinitely. On the other hand, because the silver staining is a catalytic process, the amount of silver is no guide to the amount of zinc. Danscher has estimated that as few as 10 zinc atoms can catalyze the development of a visible silver grain [13].

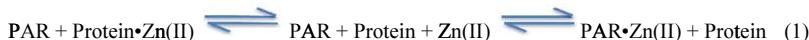
4. Determination of Macromolecule Zinc Ion Affinity

4.1 Principle and Background

For many purposes it is useful to know the affinity of macromolecules for binding zinc ion (and other metal ions), particularly of proteins. Many proteins bind zinc with varying affinity, not just those which bind zinc in a structural role such as zinc finger proteins, or in a functional role like zinc enzymes such as carbonic anhydrase or lactate dehydrogenase [14] [15]. The presence of significant concentrations of a zinc-binding protein in a medium (albumin in serum is a well-known example) creates a buffering effect where zinc ion introduced or secreted into the medium mainly becomes bound to the protein and only a minority is actually free to act elsewhere in the system. Thus it may be useful to determine the zinc affinity of such zinc-binding macromolecules

under some conditions, in many cases without a spectroscopic assay or functional assay to determine the zinc-bound form. Thus a relatively convenient colorimetric means of determining zinc affinity for otherwise cryptic proteins and other macromolecules has been developed [16, 17].

The basis of the assay is equilibrium dialysis of a protein sample against a range of suitable zinc buffers, assuming that the chelator and the protein directly compete for the metal ion (Eq. 1) followed by determination of the fractional saturation of the binding site(s) with zinc by denaturing the protein and quantifying the released zinc by absorbance using the indicator 4-(2-pyridylazo)resorcinol (PAR).



This indicator is not specific for zinc ions and can also be used to measure the concentration of Co(II), Ni(II) and Cu(II)[18]. The fractional saturation of the protein binding sites is calculated from the ratio of the bound metal ions to the total protein concentration. The K_D is calculated from a fit of a binding isotherm (Eq. 2) to the measured dependence of the fractional saturation of the protein binding site on the free metal ion concentration

$$[\text{P-Zn}] / [\text{P}_{\text{tot}}] = C / (1 + K_D / [\text{Zn}]_{\text{free}}) \quad (2)$$

where P-Zn , P_{tot} , and Zn_{free} respectively denote the metal-protein complex, total protein, and free metal ion; K_D denotes the metal dissociation constant, and C reflects the metal/protein stoichiometry at saturation.

4.2 Procedure

Plasticware should be rinsed in dilute nitric acid and rinsed with highest purity (double distilled or better) water. Micromolar concentrations of the protein in aliquots are dialyzed versus a large excess of a range of zinc buffers subtending several orders of magnitude above and below the suspected K_D : if nanomolar affinity is anticipated, buffers ranging from picomolar to micromolar free zinc should be used. The buffer volume should be larger enough that binding of zinc ions to the protein will not significantly affect the total concentration of zinc. Additionally, the dialysis should be repeated with zinc buffers where the free concentration of zinc is constant but the concentration of metal chelator is altered to test that the protein competes with the chelator for binding metal without the formation of a ternary metal:chelator: protein complex. Multiple exchange steps using Centricon filtration devices could also be used to equilibrate the protein with the zinc buffers if the kinetics are sufficiently rapid. Metal ion buffers can be formulated with existing programs such as MINEQL+ [17, 19], or published recipes may be used [20]. The combination of the high total metal concentration and the high affinities of the ligands used for metal ion buffers make it unnecessary to use specially purified reagents or chelating chromatographic resins such as Chelex-100 (Bio-Rad) to avoid metal ion contamination. The metal ion solutions should be prepared using either ICP-MS or atomic absorption metal ion standards: e.g., Aldrich cat. 18827 or similar. Note that the dialysis samples may take days to come to equilibrium depending on the kinetics of the metal ion binding [21]. Following dialysis, free metal and the metal ion buffer are rapidly separated from the protein solution by

gel filtration on a PD-10 column. This step requires that the metal dissociation rate constant is slow relative to the time required to run the column, which is generally true for tight binding. A denaturant, such as 4 M guanidinium hydrochloride, is added to the protein solution, incubated for a few minutes, and then 4-(2-pyridylazo)resorcinol (Aldrich 17,826-8) is added to a final concentration of 100 μ M (at least ten-fold molar excess over presumed metal ion concentration). The absorbance spectrum of the sample is measured between 300 and 600 nm and compared with a standard curve constructed from absorption spectra of 0-10 μ M zinc plus 100 μ M PAR in guanidinium hydrochloride.

4.3 Results

Figure 2 depicts the absorption spectrum of PAR in the absence and presence of zinc, showing the decrease in absorbance at 420 nm and the increase at 500 nm as the zinc becomes bound; a calibration curve may be constructed from the zinc-dependent changes in absorbance [22].

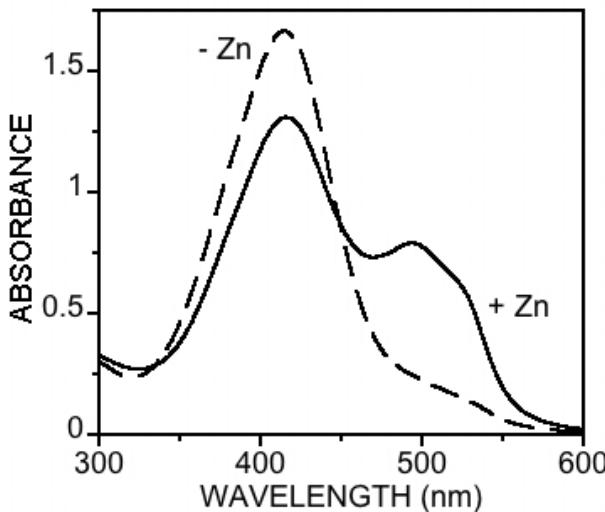


Figure 2. Absorbance spectra of PAR in the presence (solid line) and absence (dashed line) of zinc ion.

Figure 3 depicts an example of fractional saturations as a function of free zinc ion concentration for the lower affinity CA variant H119N, and the best fit to the data.

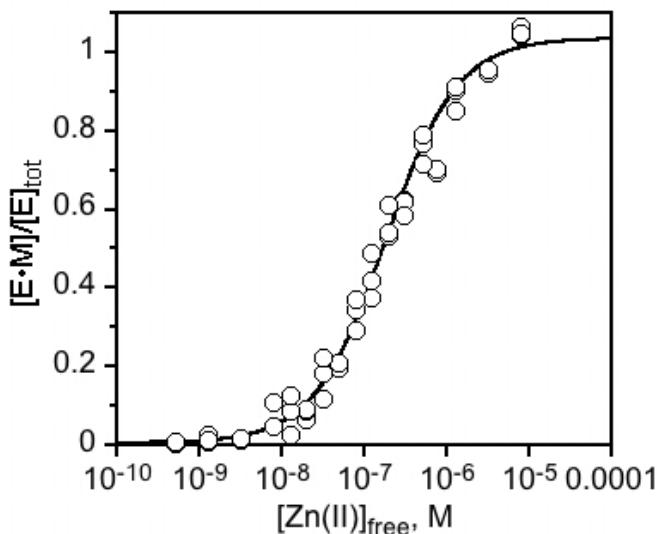


Figure 3: Fractional saturation of the low affinity human CA variant H119N as a function of free zinc concentration (circles), together with the best fit (line) $K_D = 3 \times 10^{-7} M$.

4.4 Issues

Probably the main advantages of the method are its general nature, both in terms of proteins and the metals that may be used, and its simplicity. Thus it can be used to quantify the affinity of proteins (or other macromolecules, in principle) even when their function is unknown and no activity assay is available. McCall and Fierke [18] have demonstrated the approach for Co(II), Ni(II), and Cu(II), as well as Zn(II); and extended the approach to Cd(II) and Mn(II) using a fluorescent indicator, Fura-2. Of course, other means of assaying the free metal are available, such as atomic absorption and emission spectroscopy as well as mass spectrometry and electrochemical assays, but these techniques all require complex, less common instruments and persons skilled in their use. Moreover, some of these techniques may not be compatible with relatively high guanidinium concentrations. By comparison, absorption spectrophotometers are very common and even most students are skilled in their use. The relevant portion of the PAR spectrum in use peaks near 500 nm, where relatively few biomolecules absorb and scattering is less of an issue. The method is fairly sensitive using PAR (nanomoles), and can be more sensitive if fluorescent indicators are used that are compatible with guanidinium[22]. While the method is not fast it is also not labor intensive and lends itself to multiple samples. The accuracy of measurements is good, certainly adequate for most purposes. Obviously strongly colored samples will be an issue if they overlap the absorbance (or emission) of the PAR or other indicator. Note that impure samples are not an issue unless the impurity is also a zinc binder, and for some purposes it is better to know the aggregate zinc-binding affinities of the constituents of a medium anyway.

5. Determination of Free Zinc in Specimens by Fluorescence in the Stoichiometric Regime

5.1 Principle and Background

Frequently, it is necessary to determine free zinc ion concentration (or total zinc ion concentration, following treatment of the sample) in small liquid samples. For instance, small volume liquid samples are acquired using dialysis procedures in the brains of experimental animals [23], or spinal taps to obtain cerebrospinal fluid, in addition to other specimens. These samples may contain nanomolar up to nearly millimolar concentrations of free zinc, and may or may not contain other ligands that buffer the free metal ion concentration. In some cases, it is difficult to ascertain the free zinc concentration using a fluorescent indicator or probe in an “equilibrium binding mode”: e.g., adding a fluorescent indicator with a K_D near the free zinc ion concentration to the solution such that the fractional saturation Θ of the indicator binding site reflects the free zinc ion concentration according to Equation 3:

$$\Theta = [\text{Zn}^{2+}] / [\text{Zn}^{2+}] + K_D \quad (3)$$

Equation 3 is true only if adding the indicator (generically, a ligand) does not perturb the free zinc concentration significantly, and in a small sample or with a relatively high (micromolar) concentration of indicator this is unlikely since a significant proportion of the total zinc will now be bound. More often, the affinity of the indicator is relatively high (nanomolar or tighter), and a significant concentration of the indicator must be present for its (usually optical) signal to be measured with adequate precision and accuracy. Thus, the indicator (probe) concentration is well above K_D . Under these conditions, termed the “stoichiometric regime” [24], the indicator binds essentially all the available free zinc ion, and its concentration is determined by the (small) proportion of indicator with bound zinc ion. Note that in a sample where significant concentrations of zinc ligands are present (e.g., the cell interior, or in serum) the free zinc concentration is less perturbed by the presence of the indicator, which essentially competes with the buffering ligands [25]. Unlike the equilibrium binding regime, where the plot of fractional saturation as a function of free zinc concentration describes a hyperbola, in the stoichiometric regime the fractional saturation rises linearly with zinc concentration until the binding site(s) is/are saturated (figure 4). Most often, the indicator is in excess and the experimental task is to accurately determine the very small proportion of the indicator with the metal bound.

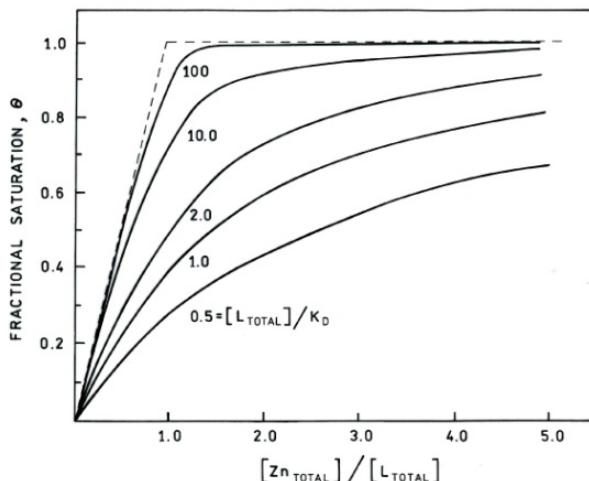


Figure 4: Fractional saturation θ as a function of the ratio of total (free + ligand-bound) zinc ion concentration present to total ligand (e.g., indicator) concentration. As total ligand increases above K_D , one approaches the stoichiometric regime (dashed line) where all free zinc is immediately bound by the excess ligand, and the concentration of the complex increases linearly with zinc concentration until the ligand is saturated. Redrawn from Weber (1992).

5.2 Procedure

The calibration curves (figure 5) for the ratiometric indicator apoCA using a 5-(dimethylamino)naphthalene-1-sulfonamide (dansylamide, or DNSA) reporter [26] were obtained with micromolar apoCA and DNSA concentrations and zinc dilutions across a 5 decade range. The right panel covers a low range from the irreducible blank of “0” zinc added (about 0.2 nM) to 10 nM of zinc added, and evidently the curve is quite linear (see [23] for more details of method).

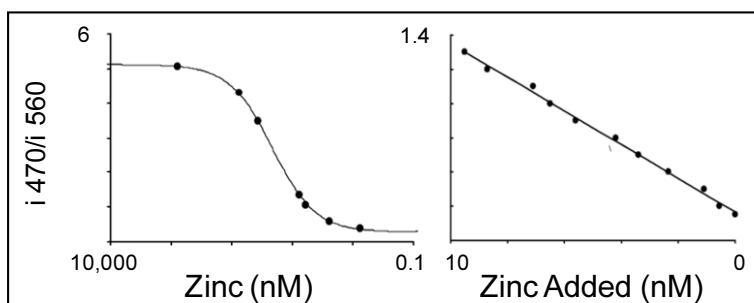


Figure 5: The left curve shows the ratiometric fluorescence result in calibration curves over 5 decades of zinc concentration (left) and over about the lowest 1.5 decades of zinc concentration (right). Note the different Y axis scales.

Measurements of “free” zinc in the extracellular fluids of the brain were made using the apoCA/DNSA system. In this work the apoCA was put into the dialysis fluid which was then run through a microdialysis probe in the brain of a rat. The free zinc entering the dialysate was “captured” by the high affinity apoCA ($K_D \sim 4$ pM) in the

dialysis tube so that simply adding the reporter allowed for fluorimetric analysis of serial aliquots of the dialysate.

5.3 Results

When we included the apoCA in a dialysis fluid and perfused rat brains by microdialysis, the DNSA indicator showed changes in the release of zinc from the brain. Nitric oxide is known to release zinc from ligands such as metallothionein by nitrosylation of the thiol zinc ligand [27]. An example of release of zinc obtained by back-dialyzing a NO* donor (Spermine NoNoate) in the brain is shown below (fig. 3; Frederickson, Prough, Thompson, Suh, unpublished).

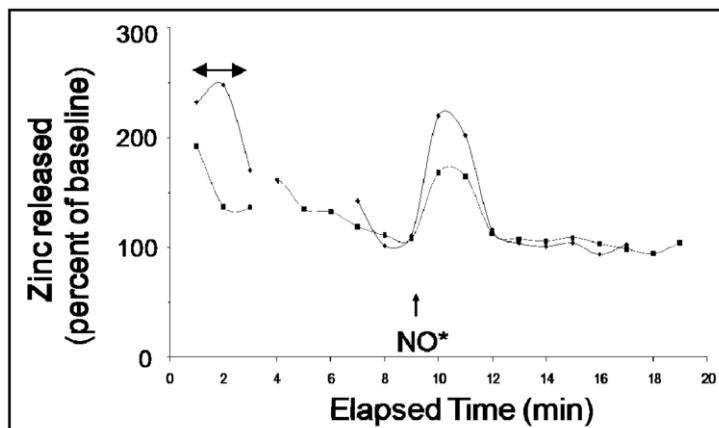


Figure 6: Zn release from rat brain following treatment with nitric oxide. After rinsing the probe (double arrow), a microdialysis probe was inserted into the brain of an anesthetized rat, allowed to stabilize, then spermine-nonoate was back-dialyzed into the brain, causing zinc efflux into the dialysate. Dialysate samples were analyzed at one minute intervals; two experiments are shown.

5.4 Issues.

The advantages of the approach are its simplicity and wide applicability. Moreover, the use of high affinity indicators in excess is desirable to trap the metal ion in experiments such as dialysis experiments where the substantial surface area of the dialysis apparatus may bind the analyte. The obvious limitation of this approach is that one must use a large molar excess of a zinc probe that has also a very high affinity. In physiological situations, adding a superabundance of high affinity probe would upset the existing speciation of the zinc amongst the endogenous ligands and (further) remove the zinc ion from any physiological signal role.

6. Imaging Free Zinc in Cells with Small Molecule Indicators (Probes)

6.1 Background and Principle

The most widely used method for imaging free zinc ion in cells employs small molecule indicators (also called probes or sensors). These indicators generally bind the

zinc ion reversibly in some chelating structure, which induces a change in fluorescence which may be observed in the microscope. Scores of such indicators have been described, and some are commercially available; they have been reviewed [28-32]. Some of the more widely used include Zinquin [33], TFL-Zn [34], FluoZin-3 [35], the ZinPyr family [36], and Zn-AF2 [37]. Most of the indicators described are “turn-on” sensors which exhibit an increase in fluorescence upon binding zinc; in the microscope, the cells (or organelles therein) with elevated free zinc concentration selectively light up. Several of these work by having zinc when bound to amine and pyridyl moieties “tie up” the nonbonding electrons on the nitrogens, which prevents the electrons migrating to quench the fluorescent moiety of the indicator, often a fluorescein derivative [38]. More recently, indicators have been devised by several groups which are “ratiometric”: the zinc binding results in a change in intensity at multiple excitation or emission wavelengths, such that the ratio of intensities varies smoothly with zinc concentration [35, 39, 40]. Correlating simple intensity changes with zinc ion concentrations (or any analyte) is viewed as impractical because of the difficulty correcting fluorescence intensity measurements for differences in cell thickness, dye loading, photobleaching, and other optical factors [41]. In a few cases fluorescence lifetimes change with zinc binding [31] (Robison, et al., unpublished results), which confers the same advantages as ratiometric measurements. For measurements in cells, it is necessary for the indicator to enter the cell, usually by being fairly hydrophobic. Some indicators are configured as acetoxymethyl esters so that they are hydrophobic initially and enter the cell, but esterase activity within the cell hydrolyzes the ester(s) to carboxylic acids, which are too polar to cross the cell membrane without benefit of a transporter protein (like the MDR multi-drug resistance transporter) and are thus trapped in the cell.

6.2 Procedure

The procedure for using these indicators is simplicity itself: the cells or tissue in a dish or microwell plate are treated with a solution of the indicator at micromolar concentration in some relatively polar solvent such as DMF or DMSO for a few minutes to $\frac{1}{2}$ hour, then the indicator solution is washed away. Fluorescence is observed in the cells with a fluorescence microscope with suitable filters. For ratiometric indicators one takes photographs successively at the two emission (or excitation) wavelengths and then electronically determines the ratio pixel by pixel; software is widely available for this purpose, e.g., IPLab from Scanalytics, Fairfax, VA. Depending on the particular indicator and the cell type, it may be necessary to subtract background from one channel or both, with ratiometric indicators. We have found Phenol Red sometimes contributes substantially to background fluorescence, so we avoid it; note that serum added to growth media often has fluorescent impurities as well as substantial and variable zinc concentrations. Excitation time and intensity should be minimized to limit photobleaching. We have found fluorescent latex spheres (Invitrogen FluoSpheres) useful as standards in monitoring microscope performance over time, since excitation sources and filters degrade over time. Temperature and buffer conditions usually have significant effects on cells and tissues, so should be controlled. The response of the indicator to varying free zinc ion concentrations is best calibrated in the microscope with metal ion buffers [17, 20, 42] in view of the difficulty of otherwise preparing low concentrations of free zinc ion reproducibly.

6.3 Results

Figure 1 depicts the mossy fiber boutons of a rat hippocampus stained with ZinPyr-1; note the clear definition of the bouton zinc.

6.4 Issues

There are many advantages and disadvantages to this approach: note that many also apply to protein-based fluorescent indicators, including those described in the next section. As we have said the approach is fast, easy, and with ratiometric probes, quantitative. Issues of concern include the affinity and selectivity of the indicator, the time to achieve binding equilibrium, the concentration of the indicator in cells, targeting the indicator to organelles, and the possibility of nonspecific binding. While the older, iminodiacetate-based (Fura family) indicators have significant affinity for calcium ion that must be somehow corrected for [43], the indicators with di-2-picolylamine (e.g., Newport Green, ZnAF series, ZinPyr-series) or tetraazacyclododecane (e.g., Rhodafluor-2) zinc binding moieties generally have high selectivity over Ca and Mg [44] and affinities in the nanomolar range or below. Kay's group was instrumental in sensitizing the community to potential interference from copper and iron as well. We note that the way to test selectivity is to measure the apparent change in affinity of the indicator in the presence of the potential interferent, not merely to assess the fluorescence response of the interferent itself [31, 44]. The time to achieve equilibrium is also of interest depending on the speed of the process to be observed. Since the affinity constant is essentially the ratio of association and dissociation rate constants (often called on- and off-rates, respectively), even an on-rate as fast as diffusion-controlled ($\approx 10^8 \text{ M}^{-1} \text{ sec}^{-1}$ for small ions in water at 25 °C.) implies 10^{-1} sec^{-1} for an off-rate for an indicator with nanomolar affinity, which will thus take some seconds to equilibrate at that concentration in the absence of catalysis. The ZinPyr family's kinetics were slower than desirable for some experiments ($K_{\text{ON}} \sim 4 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$, and $K_{\text{OFF}} \sim 3 \times 10^{-3} \text{ sec}^{-1}$, resulting in equilibration half-times of a couple of minutes [45]); presumably the rates are similar for other indicators using the di-2-picolylamine binding moiety. Recognizing this they developed faster indicators (the QZ series), albeit with micromolar affinities [45]. Another major concern is the actual amount of indicator present in the cell. Dinely, et al., showed that trappable indicators especially may accumulate to millimolar levels in cells, which not only may compromise the measurement but contribute to toxicity as well [46]. The best known zinc indicator which exhibits organelle specificity is RhodZin-3, which like other cationic dyes such as Rhodamine 123 localizes in the (energized) mitochondrion [47], although we would say its 65 nM affinity was somewhat above the physiological range. Recently, another approach which couples ZinPyr indicators to targeted proteins has been described [48]. Finally, an underappreciated issue is the probability of the indicator binding to another constituent of the cytoplasm (e.g., a protein) with an associated fluorescence change that mimics zinc binding. Of course, fluorophore binding to macromolecules such as proteins with associated increases in quantum yield and spectral shifts have been known for decades [49, 50]; of particular concern are reports of fluorescein-type dyes exhibiting quantum yield increases in injured neurons (Frederickson, Prough, et al., unpublished); a simple but seldom used test for such "non-specific" binding is measurement of fluorescence polarization (anisotropy). While the foregoing issues are of greater or lesser concern for individual experiments

depending on the goal, they are worth bearing in mind when considering the design of experiments.

7. Imaging Free Zinc in Cells by Fluorescence Excitation Ratio Using a Carbonic Anhydrase-Based Indicator: Expressed and Expressible

7.1 Background and Principle:

This approach is also designed for imaging of free zinc levels by optical fluorescence microscopy in living cells. For reviews of fluorescent zinc indicators based on human carbonic anhydrase (hCA), see [20, 51, 52]. It has been used successfully on cell lines including PC-12 (rat pheochromocytoma), CD-CHO-A (Chinese hamster ovary epithelium), ARPE-19 (human retinal pigmented epithelium), and *E. coli* DE21 to image free zinc at concentrations as low as picomolar [53] (Wang, et al., submitted; McCranor, et al., submitted). The method takes advantage of the high binding affinity and selectivity of variants of hCA for zinc ion, and transduces the binding of the metal as a change in fluorescence. The CA may be induced to enter cells by attaching a TAT-tag peptide to the sequence, or a GFP variant-CA fusion protein may be expressed in a cell or tissue if the corresponding gene is transfected into the cells. The protein variant may be targeted to particular compartments of eukaryotic cells by inclusion of an organelle targeting peptide sequence with the protein. The detection limit, selectivity, kinetics, and dynamic range of zinc determination are largely determined by the properties of the CA variant in use; for instance, the E117A variant exhibits slightly weaker affinity for zinc ion than the wild type (40 picomolar vs 4 picomolar), but much faster equilibration with zinc due to E117A's nearly thousand-fold faster association rate constant [16, 54]. The method is based on Förster resonance energy transfer (FRET) and is excitation ratiometric, meaning the free zinc concentration is proportional to the ratio of fluorescent intensities measured different excitation wavelength bands, observed at a single emission wavelength. Excitation ratios are preferred in fluorescence microscopy to obtain optimum image quality. The principle of the approach is illustrated below in (Figure 7) [55].

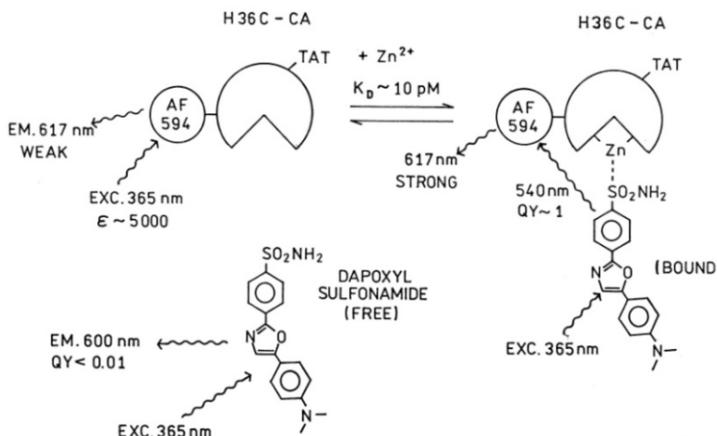


Figure 7: Principle of excitation ratiometric zinc ion biosensor

Briefly, fluorescent-labeled apoCA is introduced into the cell (see below), together with a fluorescent aryl sulfonamide, dapoxyl sulfonamide. In the presence of zinc ion, which binds to the CA active site, the sulfonamide binds to the protein at the zinc; in the absence of zinc ion the sulfonamide affinity is much lower and no binding is observed. The spectra of the bound sulfonamide and the protein label are chosen so that the former acts as an efficient FRET donor to the latter. The proportion of protein with sulfonamide bound equals the proportion with zinc bound, which in turn is a simple function of the free zinc ion concentration and the affinity. The proportion of the protein with sulfonamide bound is the ratio of the emission (at the label's wavelength, usually in the red) with excitation at the Dapoxyl sulfonamide's absorbance band (at 360 nm or so in the ultraviolet) to that at the label's absorbance maximum (usually 550 nm in the green) figure 8. The fluorescent label on the CA (a dye called Alexa Fluor 594 maleimide in (figure 7) may be replaced by a Green Fluorescent Protein homolog such as DsRed2 with suitable spectral properties by fusing the CA and fluorescent protein genes together and expressing the construct *in situ* (see below). Advantages of the approach include targeting the indicator to particular organelles by attaching targeting sequences, control of indicator levels by controlling expression, labeling subpopulations of cells by targeting transfection, and manufacture of the indicators without the need for labeling with expensive dyes or purification of the conjugate. Other groups have also described expressible zinc indicators [56-59]; however, we have much less experience with these.

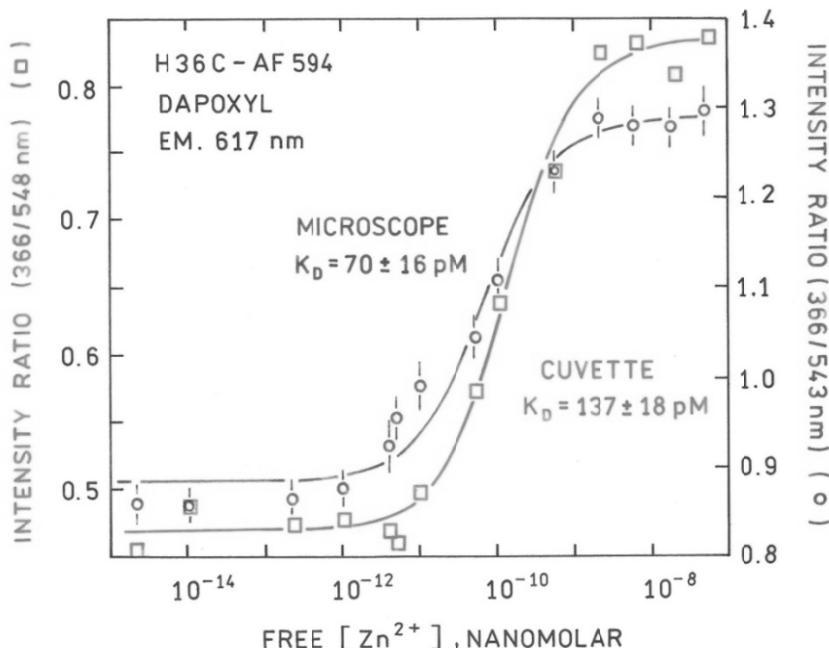


Figure 8: Calibration curves for zinc sensing determined in the microscope (circles) and spectrofluorometer (squares), together with the best fit K_D 's.

7.2 Procedure

CA II variants are fluorescent labeled using standard methods; to optimize energy transfer we typically replace an amino acid residue by site-directed mutagenesis at a position close to the active site (e.g., L198C, N67C, etc.) with a cysteine residue that can be readily labeled with a thiol-specific reactive fluorescent dye. Numerous fluorophores with thiol-specific conjugating moieties such as iodoacetamide or maleimide are available commercially from Invitrogen / Molecular Probes and other manufacturers. Fusion of CA genes with GFP homologs such as DsRed2, mCherry, or tagRFP are done using standard methods; care is necessary to assure that energy transfer will be adequate since the fluorescent protein fluorophore when attached via the CA N- or C-terminus is not as close to the Dapoxyl sulfonamide as the small molecule labels conjugated to the sites inserted into the protein sequence close to the active site. Sequences encoding TAT peptides or organelle localization signals are attached to the fused genes by standard techniques and transfection in most cell lines is done with Lipofectamine. With most cell lines TAT-labeled proteins in the surrounding medium enter within a few minutes. Dapoxyl sulfonamide (DS) is synthesized in one step from Dapoxyl sulfonyl chloride (D-10160, Invitrogen)[60] (Wang, et al., submitted); other fluorescent sulfonamides described by us and others may also be satisfactory depending upon spectral properties and lipid solubility. We customarily add dapoxyl sulfonamide in DMF at approximately one micromolar concentration to the medium surrounding the cell. The Dapoxyl sulfonamide penetrates all cells tested quickly. DS will bind not only to the labeled CA but also lipid membranes and most indigent alpha-type carbonic anhydrases; however, its fluorescence under these conditions (although enhanced) is emitted in the blue-green [60], such that it does not interfere with the (red) acceptor emission. We found that the apparent indicator response was essentially independent of DS concentration as long as the concentration was at or above the approximately micromolar K_D (results not shown). Calibration is conveniently done in the microscope with microwell plates with labeled apoprotein and dapoxyl sulfonamide with suitable zinc buffers [20]. Note that the ratio values in figure 8 are not the same for the fluorometer and fluorescence microscope because the optical properties of the two are quite different, but the apparent K_D 's differ only by a factor of two. Several cell types exhibit overt toxicity to free zinc concentrations outside a narrow range [61] so calibration in live cells in zinc-buffered media is not recommended.

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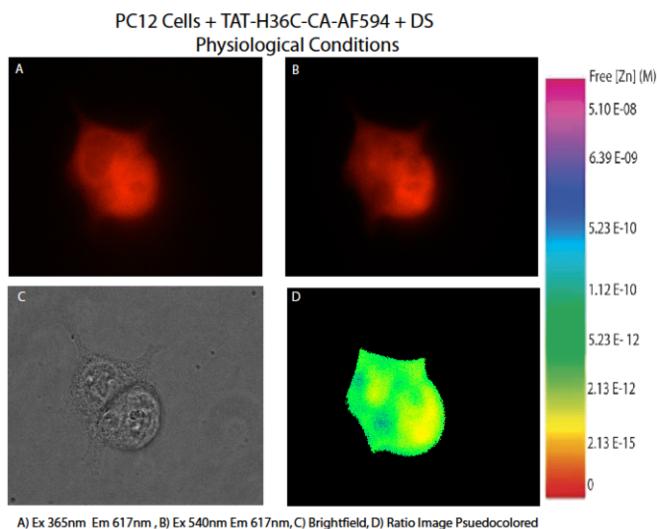


Figure 9: Micrographs of PC-12 cells taken with fluorescence emission at 617 nm, and excitation at 365 nm (upper left), excitation at 540 nm (upper right), and the resulting false color ratio image (lower right); brightfield image is at lower left.

7.3 Results

A typical false-color ratiometric image (of normal PC-12 cells), together with the original fluorescence images the ratio image was constructed from, and a brightfield image of the same cells is depicted as figure 9. The emission is far enough in the red that background is low and may be neglected. The cells were photographed in an inverted epifluorescence microscope with a 100x 1.3 NA Nikon Plan Fluor objective with a 100W Xenon arc lamp for excitation using a Cooke Sensicam QE camera. The false color image was constructed using IP Lab software using pixel-by-pixel ratioing of the other two fluorescence images.

7.4 Issues

Potential pitfalls and issues with the method include the following: In one case (a pancreatic cancer cell line) the TAT-peptide did not succeed in introducing the protein into the cytoplasm of the cell; this can be seen in the microscope by the fluorescent-labeled protein being localized in a series of puncta seeming to lie just inside the cell membrane. Some cell types are notably difficult to transfect with the expressible types. The affinity for zinc ion (and probably other cations) declines approximately one decade per pH unit [62], and wild type CA II is unstable in acidic environments below pH 6. The affinities of the wild type protein (pH 7.0) for potentially interfering divalent cations include Ca(II) (> 10 mM), Cd(II) (2.3 nM), Co (II)(150 nM), Cu(II) (0.1 pM), Mg(II) (>50 mM), and Ni(II) (17 nM); other variants exhibit different affinities for these ions [18], and other cations remain to be tested.

8. Fiber Optic Zinc Sensors for Measuring Remote or Meso-Volume Specimens

8.1 Principle and Background

In some cases the sample to be analyzed may be relatively inaccessible or inconvenient to collect: examples might include inside the living brain or deep in a body of water. In other cases the free zinc ion may be present at trace levels in a moderately-sized sample comprising a liter or less. For these applications we have developed fiber optic-based fluorescence zinc sensors which provide near real-time quantitation of zinc levels *in situ* [54, 63, 64]. The sensor utilizes a single optical fiber to carry excitation to and collect fluorescence—from the transducer figure 10. Excitation (typically from a laser) passes through a dichroic beamsplitter and is launched into the proximal end of the fiber by focusing it on the core. After passing through the fiber, it excites fluorescence at the distal end in the transducer, which is captured in part by the optical fiber and conveyed back to the proximal end, where it is not quite recollimated by the objective and reflects off the dichroic mirror towards the detector through a suitable filter. Fluorescence-based fiber optic sensors usually operate on the principle of fluorescence emission ratio or fluorescence lifetime, rather than excitation ratio or polarization (anisotropy).

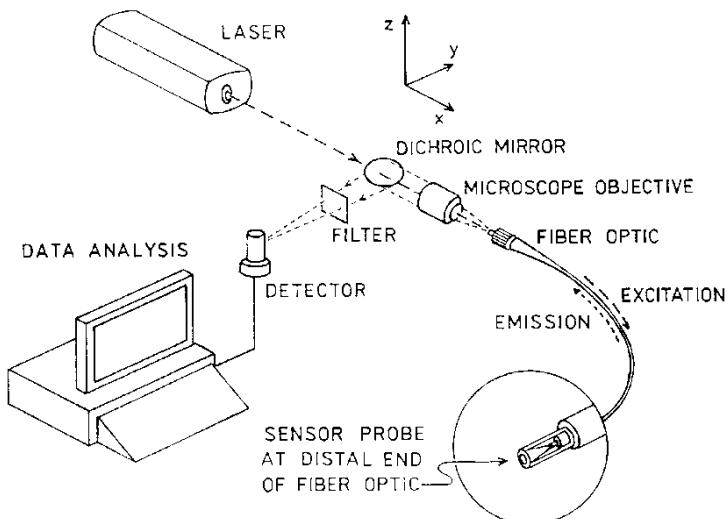


Figure 10: Single fiber optic fluorescence sensor schematic; from Thompson, et al., (2008) with permission.

The transducer itself is essentially a dialysis chamber which contains apocarbonic anhydrase (apoCA) together with a fluorescent aryl sulfonamide in polymeric form; the chamber may be a micro-chamber at the distal end of the optical fiber sealed with a dialysis membrane [26] or a hydrogel with the apoCA and polymeric sulfonamide [54] entrapped within. We note that the dialysis probes used by many investigators to study brain chemistry (including zinc ion) *in situ* provide a much slower response.

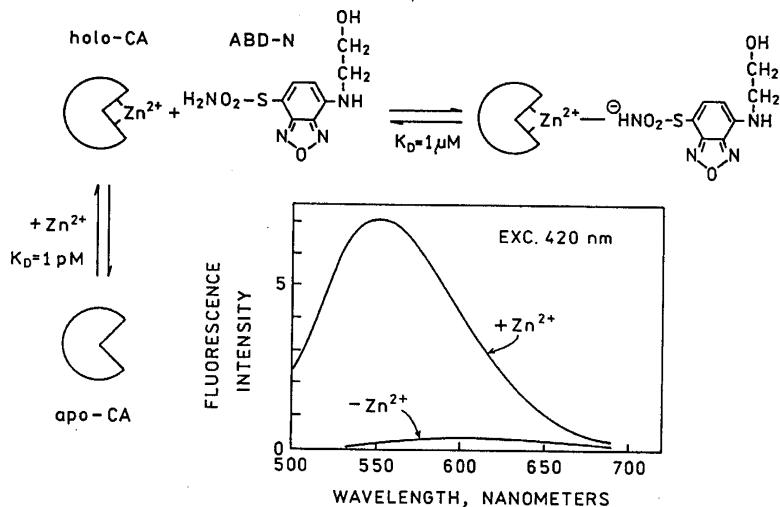


Figure 11: Principle of emission ratiometric zinc sensing with apoCA and ABDN.

This is configured as an emission ratiometric sensor, where the free zinc ion concentration is proportional to the ratio of fluorescence intensity at two different emission wavelengths figure 11. In the presence of zinc, zinc binds to the apo-CA which promotes the binding of the fluorescent aryl sulfonamide ABDN [65]. The emission of the sulfonamide when bound to the holoprotein is shifted to shorter wavelengths and increases in lifetime and quantum yield compared to the unbound form. Thus the ratio of intensities at 560 nm (bound) to 680 nm (free) is proportional to the free zinc concentration figure 12.

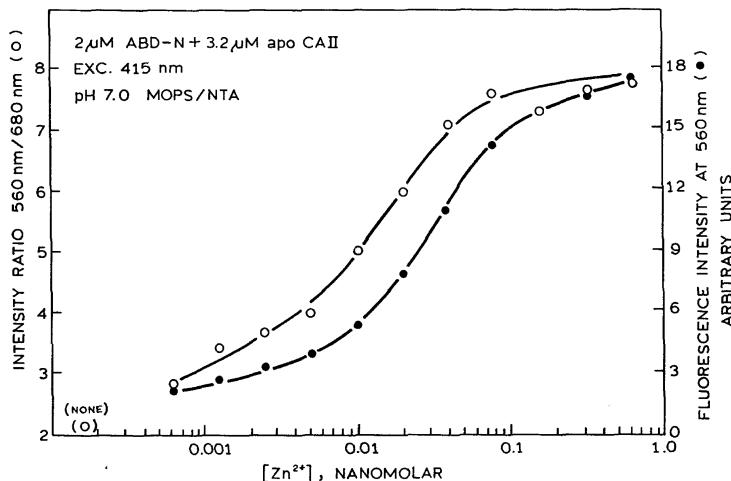


Figure 12: Free zinc ion-dependent fluorescence emission intensities at 560 nm (filled circles) and intensity ratios 560 nm / 680 nm (open circles) for apoCA plus ABDN. From Thompson, et al., 2002, with permission.

The utility of the fiber optic probe for meso-scale samples may at first be less obvious. Consider a water sample of one liter volume, containing fifty picomolar free

zinc ion concentration that is poorly buffered: e.g., there are few strong zinc ligands present, and the small fraction of zinc that is not free is essentially irreversibly bound on the time scale of the experiment. If we add one nanomolar apo-CA to the sample, more than 90% of the free zinc will bind to the protein since the free zinc concentration is well above the 4 pM K_D of the apoCA in this example. However, the 50 picomoles of zinc ion will only occupy 5% of the one nanomole of apoCA binding sites, and the signal would indicate that a much lower concentration of free zinc was present. Note that a medium like serum or serum-containing growth media with abundant, fairly strong zinc ligands with bound zinc buffers the zinc level (releases bound zinc in response to uptake by the apoCA II according to LeChatelier's principle), such that the apoCA achieves a more realistic fractional occupancy [66, 67]. Note also that this process occurs irrespective of the indicator: e.g., it is not CA-specific.

8.2 Procedure

Details of the construction of the fiber optic probes; construction, testing, and alignment of the optics; and operation have been described elsewhere [54]. Briefly, the dialysis probes are made by filling a capillary tube glued onto the distal end of the fiber with an apo-CA + poly-ABDN solution (~ 1 micromolar of each); the end of the capillary is covered with a 5 mm circular disc cut from dialysis tubing (care being taken not to introduce bubbles), which is secured with a suitable ring of Tygon or other flexible polyethylene tubing. Calibration is by immersion in suitable zinc buffers; stirring is recommended to minimize equilibration times. It is important to note that the kinetics of metal ion binding can limit the speed of response. Thus in the absence of catalysts, wild type human CA II has a dissociation rate constant for zinc of 10^{-8} sec⁻¹ [21], which means that at nanomolar concentrations of zinc at room temperature the probe will take tens of hours to equilibrate. While the temperature can be increased somewhat to speed up the equilibration, it is preferable to use a faster equilibrating variant (e.g., E117A) instead of the wild type unless the highest sensitivity is required. Excitation (in the case of poly-ABDN) is provided by a 410nm or 442 nm diode or HeCd laser.

8.3 Results

Clearly the problem with small samples is the need to have enough of the CA indicator present to provide enough signal to accurately measure the fluorescence change, without having it dramatically change the free zinc concentration. The fiber optic sensor addresses this by using a very small volume (less than one microliter) containing the fluorophore and apoCA positioned at the end of the optical fiber. If the concentration of both is a realistic one micromolar in the transducer, one has plenty of signal while only having one picomole of binding sites present, so the free concentration changes only 2% when the apoCA is over 90% bound with zinc. Examples of measurements made with such fiber optic sensors may be found in [64, 68].

8.4 Issues

Issues with the approach include the relatively poor transmission of optical fiber at the short wavelengths (400 nm) used to excite poly-ABDN, such that it is difficult to get

a good signal beyond a few tens of meters. The response time of the dialysis-type transducers (minutes) is not a good as the hydrogel-type transducers--the latter can be made quite thin (<10 micrometers) using Walt's photopolymerization technique, resulting in a quicker response [69]. At present our transducers are ~1 mm in diameter, but there seems no reason they cannot be made close to the diameter of the fiber (125 μm), if not smaller.

9. X-Ray Fluorescence for Imaging Total Zn (and Other Metals) *in situ*

9.1 Principles and Background

When atoms are excited with sufficient energy, it is possible to eject a core electron (e.g., a 1s electron). The dominant relaxation pathway for the resulting highly-excited atom is via x-ray fluorescence (XRF). For a 1s excited state, the most important fluorescence lines come from the atomic-like $2\text{p}\rightarrow 1\text{s}$ and $3\text{p}\rightarrow 1\text{s}$ transitions, giving rise to so-called K α and K β x-ray fluorescence, respectively [70]. As suggested by this description, XRF is largely an atomic phenomenon, with both the energy and the fluorescence cross-section of the K α and K β emission lines being effectively independent of the chemical environment (the small variations that do exist are orders of magnitude smaller than typical resolution of XRF experiments). In this regard, XRF is analogous to conventional atomic emission spectroscopy, with the important difference that samples for XRF do not need to be atomized. One of the key attractions of XRF is that it can be used for elemental analysis of virtually any form of matter, without the need to perturb the sample. An important corollary of this observation is the fact that *all* of the metal in a sample is detected. Thus, in contrast with zinc specific fluorophores, which are sensitive only to zinc that is "free" to bind to the fluorophore, XRF responds to all of the zinc in a sample. The combination of a zinc specific fluorophore to detect labile zinc with XRF to detect total zinc can thus provide a substantially complete description of the distribution of zinc in the sample.

X-ray emission can be observed for virtually all atoms; for elements heavier than phosphorous, there is at least one x-ray emission line that occurs in an experimentally convenient energy range, permitting versatile multi-element elemental analysis. Any excitation that is sufficiently energetic to eject a core-electron can be used for XRF excitation; this includes both energetic particles such as electrons or protons, and energetic photons. The former, sometimes called PIXE, standing for Particle (or Proton) Induced X-ray Emission, is readily coupled to electron microscopy, thus providing outstanding spatial resolution. However, for biological samples, particle excitation suffers from the need for ultra-high vacuum conditions in order to allow transmission of the particle beam, and for that reason, the focus of this section is on x-ray excited XRF. In general, PIXE is more sensitive for lower atomic number elements such as phosphorous, while x-ray excited XRF is more sensitive to heavier elements such as zinc [71].

In common with other atomic spectroscopies, XRF is potentially sensitive to matrix effects. If the element of interest is contained in a "matrix" that absorbs x-rays, this can decrease the signal, and thus the apparent concentration. For zinc in biological samples, matrix effects are generally quite small. For example, 100 μm of water, which has an x-ray absorption cross section close to that of most biological tissue, absorbs only ~7% of the zinc K α x-rays. For studies of single cells, matrix effects are thus

generally negligible. For thicker samples such as tissue, the absorption remains moderate and can easily be corrected – e.g., ~50% of the signal is absorbed by a sample that is 1 mm thick.

The sensitivity of XRF is modest on a molar basis, with typical detection limits of $\sim\mu\text{M}$. However, because this concentration sensitivity can be retained as the x-ray beam is focused to a smaller spot, the mass detection limits can be quite good. Thus, with a 1 μm x-ray spot size and a 1 μm thick cell, the irradiated volume is approximately one femtoliter and a micromolar detection limit corresponds to a zeptomole (10^{-21} mole) mass detection limit.

Although x-ray fluorescence energies show essentially no chemical sensitivity, the same is not true for x-ray absorption. For x-ray excitation energies near the absorption “edge” (the threshold energy for core-electron excitation) there can be significant energy dependent changes in x-ray absorption cross-section, raising the possibility of using XRF for chemical speciation. The changes are particularly large for redox-active elements such as selenium or sulfur, allowing one to selectively excite, for example, organic vs. inorganic selenium. By measuring XRF maps at a series of excitation energies, one can thus determine the spatial distribution of different chemical species [72, 73]. Unfortunately, zinc shows only small speciation-dependent changes in absorption [74], thus limiting the practical ability to determine speciation dependent zinc distributions.

9.2 Procedure

Since any form of matter can be studied using XRF, it would be possible to make measurements with little or no sample preparation. In practice, biological samples are often prepared by plunge freezing in an organic cryogen such as liquid isopentane, a technique that has been widely adopted by the electron microscopy community. In general, plunge freezing appears to do a good job at retaining internal structure, and in particular, at retaining metal distributions unchanged. After freezing, samples are often lyophilized giving a dry sample that is less sensitive to radiation damage. Alternative approaches involve conventional chemical fixation. These have the advantage of giving samples that are directly comparable to those used in other microscopies, but run the risk that the fixation process may lead to changes in metal distributions.

Although any x-ray source could be used for XRF, it has only become a practical technique for studying dilute metal distributions in biological samples with the development of intense synchrotron x-ray sources. Synchrotrons are large, typically national laboratories that provide intense, highly collimated (i.e. laser like) x-ray beams from accelerating a beam of electrons (for details on synchrotron sources worldwide see www.lightsources.org/cms/). For details on the worldwide availability of synchrotron radiation see e.g. reference [75]. Most synchrotron laboratories have at least one “beamline” that is equipped for XRF studies. In the best cases (i.e., the so-called third-generation synchrotron sources) the incident x-ray beam can be focused to a spot size of ~ 100 nm or smaller, allowing XRF to be used to study metal distributions in individual cells.

Samples are typically scanned relative to the x-ray beam and the resulting x-ray fluorescence for each pixel is detected using an energy-resolving x-ray detector to distinguish the fluorescence of each element. With the use of the appropriate standard reference compounds, it is possible to convert the measured x-ray fluorescence intensity directly to metal concentration. It is important to note, however, that

concentration is typically calculated in rather unusual units of g/cm^2 rather than the more conventional grams (or moles) per volume. This reflects the fact that XRF images are two-dimensional projections of the metal distribution. For many purposes, the relative distribution of an element is sufficient; if concentration is needed it is necessary to know the sample thickness.

9.3 Results

A wide variety of biological samples have been studied using x-ray fluorescence [76, 77] [78, 79]. In studies of tissue samples, much of the focus has been on tissues where zinc accumulation is known to correlate with disease. Examples include studies of the distribution of zinc in the brain [80, 81], and in sub-retinal pigment epithelial deposits associated with macular degeneration [82]. In each case, XRF was used to complement other imaging modalities, allowing demonstration that zinc correlates with the expression *in vivo* of the transporter ZnT3 [80] or that zinc and copper accumulation correlates with amyloid plaque localization, as detected by infrared imaging [81]. The ability to probe all metals at once was important for comparing zinc, copper, and iron distributions in brain tissue, and has also been useful in studies of inorganic distributions in bone [83]. Figure 13 depicts a map of zinc levels in a yeast cell, showing elevated levels in the nucleus and vacuole (Penner-Hahn, et al., submitted).

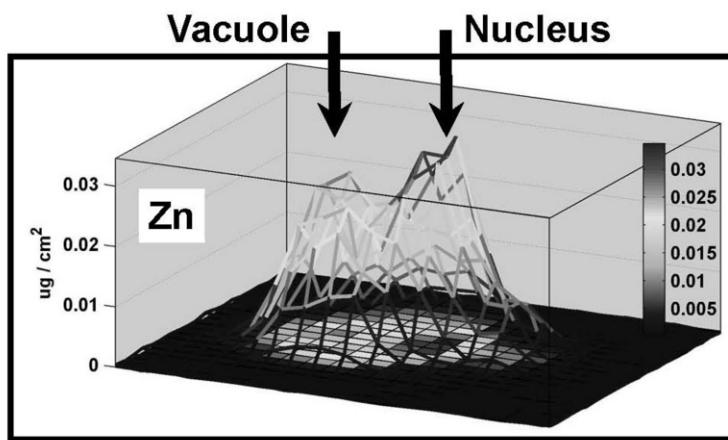


Figure 13. XRF-detected zinc distribution in $\mu\text{g}/\text{cm}^2$ for a budding yeast cell (Zahler, Fierke, Kidd, and Penner-Hahn, unpublished results). The location of the vacuole and nucleus were determined using GFP and DAPI (4',6-diamidino-2-phenylindole) fluorescence, respectively.

While XRF has been used far less than other imaging modalities to study zinc in cells, there are an increasing number of studies that use XRF to map the distribution of zinc (and typically other elements) in cells. For example, a recent study of developing oocytes revealed a 50% increase in total zinc during meiotic maturation [84]. As with the tissue studies, it is often advantageous to combine XRF with other imaging modalities. For example, previous work using zinc fluorophores had shown that deletion of the ZnT3 protein from presynaptic vesicles eliminates the presence of histochemically stainable zinc. However, this leaves open the question of whether these cells contain less total zinc or simply have less bioavailable zinc. XRF mapping demonstrated that it is, in fact, the total zinc concentration that decreases[85].

9.4 Issues

Perhaps the greatest strength of XRF is its ability to probe the spatial distribution of all of the metal atoms in a sample with minimal sample perturbation. In principle, aside from limits on detectability, all metals can be studied simultaneously, and there is no need to worry that the distribution of metals has been changed by, for example, addition of a metal chelator. As noted above, XRF detects all of the metal, regardless of chemical form. While a few elements such as Cd and Gd may require special conditions, all elements heavier than P are detectable by XRF. Well-designed experiments exploit this complementarity to imaging methods that probe labile metals.

These strengths are balanced by three weaknesses of XRF: sensitivity, two-dimensionality, and radiation damage. The intrinsic sensitivity of XRF is ultimately limited by the fact that x-ray absorption cross-sections are quite modest in comparison with typical optical absorption cross-sections for fluorophores. For the hard x-rays that are used in XRF ($\sim 1 \text{ \AA}$ wavelength), the molar absorptivity of Zn is $\sim 15 \text{ M}^{-1}\text{cm}^{-1}$, orders of magnitude less than that of the best fluorophore. This limits the sensitivity and, perhaps more importantly, tends to make XRF imaging quite slow. A fluorescence nanoprobe image of a single cell at $\sim 150 \text{ nm}$ resolution may require ~ 1 hour.

In most cases, XRF images are two-dimensional projections of the three-dimensional distribution of metal ions in a cell. If the sample is rotated and XRF images are measured as a function of angle, it is possible to reconstruct the complete three-dimensional distribution of metals by measuring the XRF image as the sample is rotated [86]. In practice, these are challenging measurements for many biological samples, both because of the time required and because of the possibility that the sample will change, for example from dehydration, during the course of the measurements. It is perhaps not coincidence that some of the most striking XRF tomography measurements to date have used dry samples such as seeds[87] or diatoms[88].

X-rays are ionizing radiation and thus have the potential to cause damage to biological samples, and it is unlikely that XRF could ever be used to image living samples. In order to prevent radiation damage, samples are typically studied either in frozen form or after lyophilization. These seem to be effective at preserving the internal metal distribution. It is likely that redox active metals suffer radiation damage—typically x-ray induced photoreduction – during lengthy XRF images; fortunately, XRF is not sensitive to metal oxidation state.

Overall, XRF is an exciting new imaging method that is uniquely able to provide $\sim 100 \text{ nm}$ resolution images of the total zinc (and other metal) content of biological samples. As third-generation synchrotron sources continue to develop with more beam time and easier access, XRF is likely to see continued growth.

10. Conclusion and Perspectives

After several years of work in a number of laboratories, we are now beginning to assemble a number of methods for measuring zinc ion levels and fluxes in cells, tissues, and (small) whole organisms: a toolbox for studying zinc biology. One hopes these and other methods will be adequate to resolve many questions, particularly those focusing on zinc's putative roles in human disease. The study of calcium has made clear the necessity of reliable quantitation by ratiometric, anisotropy(polarization), or lifetime-

based fluorescent indicators. The emergence of expressible sensors which may be targeted for particular organelles or tissues is especially exciting and will lead to many new discoveries. Coupling fluorescence with other imaging modalities (NMR, optical coherence tomography, photoacoustic spectroscopy, etc.) also offers interesting possibilities. Work is already underway to develop fluorescent sensors usable in large animal models. Ancillary tools are also needed: a cell-permeant zinc chelator with better specificity and fewer issues than TPEN is desirable, as well as a more specific zinc ionophore than pyrithione, and a usable "caged" form of zinc. Some examples of such tools have been described, but they need to be made commercially available. We and others have described zinc buffer formulations, but these will not be widely used until they can be purchased. While many interesting challenges lie ahead, it is clear we have come a long way, and can expect further discoveries as the tools improve.

Some years ago, one of the authors wrote a short article entitled "Studying zinc biology with fluorescence: Ain't we got fun?"; as the developments described above and in other chapters in this volume make clear, we're having more fun than ever.

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II. ZINC IN HEALTH AND DISEASE

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10. Immunobiology and Hematology of Zinc

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Abstract. The essentiality of the trace element zinc is anchored in its role as a cofactor in a number of enzymes and in its function as a signaling molecule. Among other cell systems, the immune system requires zinc for adequate functionality. Not only zinc deprivation but also exceedingly high zinc levels result in immune dysfunction, depicting the significance of a regulated zinc homeostasis for the immune response. Disturbances of zinc homeostasis affect multiple aspects of the immune system, including hematopoiesis, innate immunity, adaptive immune response and processes involved in immune regulation. Zinc deficiency impairs the development of lymphocyte progenitors and modifies the differentiation of cells of the innate immune system. Furthermore, immune cell function is adversely affected during zinc deprivation, resulting in compromised lymphocyte immune reaction combined with dysregulated innate immune responses. Consequently, the development and progression of various infections and diseases is influenced by alterations in the zinc homeostasis. For several decades, the immunobiology of zinc has been studied. This chapter aims to discuss the overall impact of zinc on the immune system.

Keywords. Adaptive immunity, cytokines, hematopoiesis, innate immunity, zinc deficiency

Introduction

The trace element zinc is essential in a variety of cellular functions involving all organ systems. Its ubiquity emphasizes its importance in cell systems, which is reasoned by the crucial role of this ion as cofactor of more than 300 enzymes. Particularly transcription and replication factors require zinc for their structural integrity, e.g. in zinc finger motifs [1; 2]. The more severe are the consequences during zinc deficiency, causing a dramatic loss of these systems' functionality. In mammals, zinc deficiency particularly comes apparent in its effect on strongly proliferating cell systems such as the skin, hair, reproductive organs and the immune system [3; 4]. This chapter will concentrate on the effects of zinc on hematopoietic processes and the immune system, being of the utmost importance for almost every multicellular organism.

Zinc homeostasis is tightly controlled by several mechanisms including the expression of zinc transporter (see more details in chapter 8). Disturbance of the cellular zinc availability, either by zinc deficiency or by a dysfunction of zinc

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metabolism, has been known to compromise immune function [5-8]. Remarkably, zinc does not affect only a single component of the immune system. Rather it influences the expression of several hundreds of genes in immune cells. This complexity is reflected in the functional consequences of zinc deficiency, which act on multiple cross linked levels. Lymphopoiesis, virtually all types of mature immune cells, cytokine production, and the polarization of T helper subsets are all affected by zinc deficiency, to mention only a few examples [9]. Based on these functional consequences, zinc deficiency is associated with allergic, autoimmune and infectious diseases (see chapters 11-13).

Above all, the rare autosomal recessive inheritable disease *acrodermatitis enteropathica* is a consequence of zinc deficiency due to a mutation of the intestinal zinc uptake protein Zrt/Irt-like protein (Zip) 4. The resulting zinc-specific malabsorption syndrome leads to several immunological alterations such as thymic atrophy, decreased lymphocyte counts and function, and death from infections. However, pharmacological zinc supplementation can reverse most of the disease's symptoms [10]. Besides, a decline of immune function in marginally zinc-deficient elderly people was demonstrated, which can be improved by restoration of zinc levels via zinc supplementation [11]. According to these observations, the importance of zinc for the integrity of the immune system is out of question.

Furthermore, experiments in mice even indicated zinc-associated epigenetic effects. In particular it was shown that gestational zinc deficiency not only impaired the immune function of the offspring of the parental mice, but also compromised immune function even in the second and third filial generation, whose diet contained a sufficient supply of zinc [12]. The impact of zinc is partially owed to its crucial role as a signalling ion, acting on immune cell signal transduction and resulting in the alteration of gene expression, which is further discussed in chapter 6.

Not surprisingly, the World Health Organization (WHO) has identified zinc deficiency as one of the leading risk factors of disease, ranking fifth in developing countries and eleventh in developed countries [13]. This alarming finding justifies the intensive research in this field within the last few decades and underlines the needs for ongoing efforts.

1. Zinc and Hematopoiesis

Hematopoiesis entitles the generation of a wide variety of distinct blood cell types from a common hematopoietic stem cell (HSC). Blood cells can be subdivided into two major subgroups: myeloid and lymphoid cells. Whereas the myeloid blood cells consist of erythrocytes, megakaryocytes, mast cells, granulocytes and monocytes/macrophages, the lymphoid cells include B cells, T cells and NK cells. The differentiation into lineage specific immune cells comprises sequential stages of development during hematopoiesis. In mature adult mammals, all blood cells derive from bone marrow residing HSCs. Whereas the myeloid progenitor cells develop in the bone marrow, bone marrow hematopoietic stem cell-derived precursors further migrate into the thymus where they undergo subsequent maturation. Remaining lymphoid progenitors in the bone marrow develop into B lymphocytes and natural killer (NK) cells. Due to their central role in leukocyte development, the bone marrow and the thymus are also referred to as primary lymphoid tissue.

Given the fact that zinc plays a key role in cell division and replication (see chapter 5), an adequate supply of zinc can be assumed to be essential for proper hematopoietic

processes. Indeed, investigations in rodents revealed that zinc deprivation induces lymphopenia and thymic atrophy due to high losses of lymphoid precursors caused by apoptotic cell death [14].

Developing thymocytes pass through different stages of maturation in the thymus. T cell progenitors, derived from the bone marrow, mature into double negative T cells ($CD3^+CD4^-CD8^-$). In these cells the rearrangement of the T cell receptor (TCR) begins, and subsequently, CD4 and CD8 molecules (double positive T cells) as well as low levels of TCR are expressed. Positive and negative selection occurs at this stage. Later, positively selected cells develop into single-positive T cells, carrying either $CD4^+$ or $CD8^+$, and enter the periphery.

The impact of zinc deficiency in thymopoietic processes was demonstrated to affect particularly pre-T cells, since a preferential loss of pre-T cells was found in chronically zinc-deficient mice as well as in acute zinc-deficient mice [14]. The loss of pre-T cells was related to an accelerated amount of apoptosis in these cells, consistent with the finding that pre-T cells express the lowest amount of the anti-apoptotic proteins Bcl-2 and Bcl-XL, thereby increasing their vulnerability towards cell death. Since the thymus comprises about 80% pre-T cells, its susceptibility towards zinc deficiency is comparatively prominent. Zinc deficiency-induced apoptosis correlates with an increasing amount of the steroidhormone glucocorticoid, which is suspected to mediate cell death [15].

In contrast to thymopoiesis, acute and chronic zinc deficiency affect B cell lymphopoiesis in different ways. Following acute zinc deficiency, the B cell compartment of young adult mice is largely reduced and the composition of surviving B cells within the B cell compartment markedly changed [14]. Analogously to T cell maturation, developing B cells also undergo a series of distinct phases. Early pro-B cells show the first rearrangement of immunoglobuline (Ig) genes and express a reasonable amount of the anti-apoptotic protein Bcl-2. Large pro-B cells, expressing a pre-B cell receptor (BCR), mature into pre-B cells, which upon completing Ig rearrangement, express surface BCR molecules. Pre- and immature B cells provide a certain degree of danger to the body after completing BCR gene rearrangement, as they might express molecules that do not fulfill their assigned function leading to interaction with self-antigens. These cells are usually eliminated apoptotically. Cells of these lineages therefore express only marginal amounts of Bcl-2 and are hence jeopardized during acute zinc deficiency [16].

Mature B cells express both types of BCR, IgM and IgD, and migrate into the periphery. Here, they develop into naive B cells, ready to exert antibody-mediated responses. Due to their strong expression of Bcl-2, naive B cells are only marginally prone to apoptosis. Similar to pre-T cells, after suboptimal zinc dietary intake both pre-B and immature B cell numbers are markedly reduced (about 50% to 70%). Chronic zinc deficiency, on the other hand, showed no effect on B cell lymphopoiesis. Moreover, during acute zinc deficiency, cells of the myeloid lineages increased both in proportion and absolute number, especially the myeloid compartments containing monocytic cells exhibited an 80% increase. In spite of enhanced myelopoiesis during acute zinc deficiency, the myeloid lineages remained intact during chronic zinc deficiency [14]. Considering the fact that cells of the myeloid lineage represent the first line of defense, protecting these cells during acute zinc deficiency appears to be pivotal. Interestingly, a recent study revealed an impact of the zinc level on the differentiation of monocytes [17]. The commitment of $CD34^+$ hematopoietic stem cells to the monocytic lineage requires physiological levels of the steroid hormone 1, 25-dihydroxyvitamin D3

(1,25D3). It was observed that during 1,25D3 mediated differentiation a reduction of the free intracellular zinc level, measured by Fluozin-3 [18], occurred in the acute myeloid leukemia cell line HL-60. Reduced gene expression of several zinc transporters, predominantly of the so-called Zip-family (zinc importers), and enhanced expression of the zinc binding proteins S100A8 and S100A9 was reported. Decreased free intracellular zinc level may therefore be due to a diminished uptake of extracellular zinc and enhanced zinc sequestration. Moreover, reduction of the intracellular zinc by using the zinc chelator N,N,N',N'-Tetrakis(2-pyridylmethyl)-ethylenediamine (TPEN) or using zinc depleted cell-culture medium enhanced 1,25D3 mediated differentiation [17]. Hence, the reduction of intracellular zinc after 1,25D3 treatment promotes monocyte differentiation and highlights cellular zinc homeostasis as an endogenous modulator of monocyte differentiation. The above findings confirm the idea that low zinc levels establish a favorable environment for monocyte development. Thus, hypozincemia, as found during the acute-phase response, can be suggested to have a systemic signaling character, including the promotion of monocyte development. Moreover, the decline in the number of the lymphoid cells during zinc deprivation is compensated by the substantial increase of the myeloid compartment, which leads to the suggestion that "reprogramming" of the immune response allows for a partial preservation of the innate immune response.

Whereas acute zinc deficiency causes reprogramming of the immune system, chronic zinc deficiency seems to enable sufficient adjustment to the low zinc level to maintain B cell lymphopoiesis and myelopoiesis for a prolonged time period. This is congruent with the finding that chronically zinc deficient mice did not develop alopecia, parakeratosis or any external evidence of zinc deficiency, suggesting that adaption to slowly decreasing zinc levels can be obtained in the bone marrow [14]. Another study conducted in moderate zinc deficient humans support the idea, that mild zinc deficient individuals are able to maintain lymphopoiesis and hematopoiesis for an extended period of time, since the subjects did not have altered peripheral blood mononuclear cell (PBMC) phenotypes [19].

In addition, the effect of zinc deficiency on the cell count of white blood cells (total white blood cells, granulocytes, monocytes and lymphocytes) was investigated in rats [20]. The results confirmed the aforementioned findings in that the cell count of the myeloid lineage increased during zinc deficiency. However, in sharp contrast to the aforementioned results in mice, this latter study did not reveal any change in the number of peripheral lymphocytes as well as unaltered numbers of blood lymphocyte subsets. The diverging results may be due to the fact that the zinc level in these rats ranged between those measured for acute and chronic zinc deficiency in mice, respectively.

Hematopoiesis also includes the development of erythrocytes, called erythropoiesis, which takes place in the bone marrow as well. Erythropoiesis appears to be highly susceptible to zinc deficiency, reflected in that both acute and chronic zinc deficiency induces losses of up to 60% or 35% respectively in the nucleated erythroid cell population [14]. This finding complements the observation that in humans, more acute forms of zinc deficiency are accompanied by anemia [21].

Furthermore, a correlation between zinc deficiency and sickle cell disease (SCD) persists, underlying the hypothesis of zinc being an important constituent of erythrocytes. First studies in 1975 [22] reported that zinc concentrations in the plasma, erythrocytes and hair were significantly lower and urinary excretion of zinc higher in the SCD subjects compared to the normal controls. Erythrocyte zinc and daily urinary

zinc excretion were inversely correlated in the SCD patients ($r = -0.63$, $p < 0.05$), suggesting that hyperzincuria may have caused zinc deficiency in these patients. Carbonic anhydrase, a zinc metalloenzyme, correlated significantly with erythrocyte zinc ($r = +0.94$, $p < 0.001$). Plasma RNase activity was increased in the SCD subjects in comparison to the controls, and inasmuch as activity of RNAase is inhibited by zinc and thereby its activity is increased in zinc deficient tissues [23], the increased plasma RNase activity in SCD patients serves as another evidence of zinc deficiency in these patients.

During the past 35 years, many studies have now shown that zinc deficiency is fairly common in patients with SCD [24; 25]. Prasad and his group estimate nearly 60% to 70% of these subjects to be zinc deficient. Besides growth retardation and hypogonadism in males, other manifestations related to zinc deficiency include neuro-sensory disorder (abnormal dark adaptation), hyperammonemia, cell-mediated immune dysfunctions, increased oxidative stress and increased generation of pro-inflammatory cytokines such as TNF- α and IL-1 β , all of which have been reported to be responsive to zinc supplementation [26-29].

The zinc levels in plasma, erythrocytes and neutrophils were significantly decreased in SCD subjects in comparison to 25 controls including both black and whites. A significant positive correlation coefficient between the body weight and neutrophil zinc levels was observed ($r = 0.66$, $p < 0.01$). Also, a significant positive correlation between the height and neutrophil zinc levels in SCD subjects was reported ($r = 0.62$, $p < 0.01$).

In comparison to the placebo treatment, zinc supplementation for one year resulted in a significantly greater increase in height, mean \pm SD (Zn gr 6.4 ± 1.0 - vs placebo gr 2.2 ± 0.3 cm, $p < 0.001$) and greater increase in weight, mean \pm SD (Zn gr 4.4 ± 1.4 vs 0.9 ± 0.7 kg, $p < 0.001$). Plasma testosterone and activities of the zinc-dependent enzymes neutrophil alkaline phosphatase and nucleoside phosphorylate in erythrocytes also increased significantly in the zinc supplemented subjects in comparison to the placebo group [29].

In 1978 it was observed for the first time that plasma ammonia level in human volunteers increased when they received restricted zinc intake [30]. After oral supplementation with zinc, the plasma ammonia concentration returned to normal levels. This was an unexpected finding, and to that time hyperammonemia had not been related to zinc deficiency either in human subjects or experimental animals. This problem was also investigated in zinc-deficient rats [31]. An increased level of plasma ammonia and a decreased activity of hepatic ornithine carbamoyltransferase (OCT), an enzyme required for urea synthesis, were observed in deficient animals as compared to the pair-fed controls [31].

In SCD patients, significantly increased plasma ammonia, decreased plasma zinc, and decreased level of plasma urea nitrogen were observed [32]. Zinc supplementation (75 mg/d) corrected these parameters. Once the therapy with zinc was discontinued, plasma ammonia levels increased and plasma urea nitrogen level decreased which was again corrected by zinc supplementation. These observations established the effect of zinc on plasma ammonia levels in SCD subjects.

Brody et al. [33] reported an increased activity of muscle adenosine monophosphate (AMP) deaminase, an enzyme of the purine nucleotide cycle, in zinc deficient rat muscle. This finding is consistent with the observation that plasma aspartic acid levels are reduced and plasma ammonia levels are increased due to deficiency of zinc, inasmuch as the purine nucleotide cycle results in the net production of ammonia

from aspartic acid [33]. Yoshino et al. [34] reported the AMP deaminase is inhibited by zinc with a remarkably high affinity. Hence, in zinc deficiency reversal of zinc inhibition of the enzyme may contribute to the increase in ammonia level.

Thus, the studies by Prasad et al. showed that hyperammonemia and decreased blood urea nitrogen in SCD patients are related to zinc deficiency. Inasmuch as increased ammonia levels are harmful to brain functions, zinc deficiency must be corrected in SCD patients who also suffer from neurological complications.

Most patients with SCD have symptomatic painful crises which are thought to be due to red blood cell sickling and obstruction of small blood vessels. This leads to greater deoxygenation of the red cells in neighboring vessels and further sickling. In limited clinical trials oral zinc therapy resulted in over-all reduction in pain-crises frequency. Furthermore, it was observed that oral zinc therapy significantly decreased the number of irreversibly sickled cells (ISC) in some patients [35], thus providing evidence that zinc was affecting sickling process *in vivo*.

Zinc also increased the *in vitro* filterability of partially deoxygenated sickle cells [35]. During sickling red cells accumulate calcium and this may have adverse effect on the cell membrane and on cell deformability. It was also shown that zinc decreases calcium retention by red cell membranes, decreases calcium induced hemoglobin binding to red cell membranes and antagonizes the erythrocyte-promoting effect of calcium. Since zinc therapy reduces the ISC counts in many patients with SCD, further clinical evaluation of this agent is highly indicated.

Another phenomenon related to zinc deficiency is the occurrence of copper deficiency, which can be induced by excessive zinc intake. Studies in SCD patients showed that the number of ISC is substantially decreased *in vivo* following therapeutic oral administration of zinc (25 mg every 4 h) [35]. The occurrence of hypocupremia and hypoceruloplasminemia in an adult SCD patient receiving zinc therapy warranted the researchers to look for this complication in further 13 SCD subjects, who were treated with therapeutic level of zinc. Although leucopenia and microcytosis were observed in only one patient, hypocupremia developed in approximately half of the patients who received high doses of zinc for six months.

Even though the plasma copper and ceruloplasmin levels do not establish copper deficiency, the correction of microcytosis, leukocyte count and plasma copper level by administration of copper sulfate lends support to this diagnosis. Furthermore, since plasma copper levels in SCD patients are usually increased, a decrease following zinc therapy may be indicative of copper depletion.

Excess zinc is long known to produce copper deficiency in experimental animals [36]. High plasma copper levels with low plasma zinc levels have been reported previously in zinc deficient dwarfs from the Middle East and in SCD patients [37]. Thus, in experimental animals and human subjects, a reciprocal relationship between zinc and copper exists, suggesting that both elements may be competing for similar binding sites in the tissues. The biological interactions of elements are probably based on the physicochemical properties of their ions. This concept has led to the proposal that ions whose valence shell electronic structures were similar would be antagonists to each other biologically [36]. The electronic structure of the cuprous ion is d10. Zinc²⁺, Cd²⁺, and Hg²⁺ also have the same structure of the valence shell as the cuprous ion; thus these elements should prove to be antagonists to copper.

Elements such as zinc and cadmium decrease copper absorption and reduce plasma copper concentration levels when ingested at high dietary levels. The mechanisms that regulate copper absorption are little understood, although it seems clear that metal

binding components are involved and that the inhibition of copper absorption brought about by various metals results from competition for protein-metal-binding sites [36].

The study in SCD by Prasad et al. demonstrates that patients receiving zinc orally in high amounts much greater than the minimum daily requirement (15 mg/d) may deplete the body of its store of copper. This complication of zinc therapy in man appears to be easily correctable.

In recent years, zinc ingestion has become increasingly popular in the lay and food faddist population. Several cases have now been reported in patients who ingested high quantities of zinc, who developed copper deficiency sideroblastic anemia, and bone marrow depression [38].

Although excessive and oral zinc was known to produce copper deficiency in animals, Porter et al. [39] in a letter to the editor in 1977, reported this phenomena in a patient who was taking 600 mg of zinc sulfate daily for 14 months to treat celiac disease, when anemia and neutropenia developed. A year later, Prasad et al. [40] reported similar findings in patients with SCD who were receiving therapeutic levels of zinc for decreasing irreversible sickle cells.

A most interesting case was that of a 31 year old schizophrenic man who had been ingesting coins for 10 years. Profound anemia, neutropenia and virtually absent serum copper and ceruloplasmin levels with elevated zinc levels emerged [41]. Gastrectomy recovered \$22.50 worth of coins mostly pennies in various stages of digestion. After surgery, his bone marrow function returned to normal after 4 weeks. The copper deficiency was attributed to the ingestion of pennies, which since 1982 are composed of 98% zinc and 2% copper.

Anemia induced by hyperingestion of zinc was characterized by either microcytosis, normocytosis, or macrocytosis, neutropenia and normal platelet count. Bone marrow examination showed sideroblasts and some were ringed sideroblasts. Copper replacement corrected the hematologic abnormalities.

An additional approach to investigate the role of zinc during hematopoiesis was exerted in a controlled study of a rat model investigating the effect of zinc supplementation on the total-body irradiation-induced suppression of hematopoiesis [42]. The irradiation provoked a decreased production of white blood cells, which was partially reversible by zinc supplementation. The latter provided a protective effect on white blood cell count against total-body irradiation and might reduce radiation-induced toxicity in radiation-treated cancer patients.

In conclusion, zinc seems to affect hematopoietic processes in a multifold manner. One needs to differentiate between acute zinc deficiency and chronic zinc deficiency with their specific effects on the various hematopoietic cell lineages. The body seems to be able to adapt to altering zinc concentrations by reprogramming the immune system. The specific extent to which zinc exerts its functions, including-amongst others-the production of cytokines and stromal factors needs further investigations. Still, many details remain obscure, especially in what concerns the deviating effects in the innate versus adaptive immune system. The following section will give an overview of general effects of zinc; and then switches to a more detailed discussion of its function in innate and adaptive immunity, respectively.

2. Zinc and General Immune Function

The immune system can be subdivided into different parts. Innate immunity comprises the first line of defense, including monocytes/macrophages, granulocytes, dendritic cells (DC), mast cells and natural killer (NK) cells as cellular components and a number of proteins, including the complement system. Cells of this part are specialized to perform a fast immune response but, on the downside, lack immunological memory. For this reason, these cells are present as completely differentiated immune cells in the peripheral blood without undergoing further development. One of the first responses of innate immunity upon antigen exposure is the inflammatory response, involving local accumulation of fluid, plasma proteins and leukocytes.

The specific immune system, on the other hand, is formed by two parts, humoral immunity (B cells) and the cellular immunity (T cells). The specific immune system consists of precursor cells which act against specific antigens. After antigen contact the cells differentiate into effector and memory cells, thereby establishing a more efficient immune reaction due to their future fast recognition of a given antigen and subsequent immune cell activation. Zinc is known to influence both parts of the immune system in a multi-faceted way; nonetheless, adaptive immunity, particularly T cell response, is most prominently affected by zinc.

Since zinc strongly influences proliferating systems [3], not only the immune system is zinc dependent, but also proliferation and expansion of pathogens is affected by the surrounding zinc status in the host [9]. Thus, decreasing zinc concentrations in the plasma by the human body represents one acute phase response during infections. Hypozincemia is induced via chelation of zinc by the zinc and calcium binding S-100 protein calprotectin, which is released by leukocytes. Calprotectin suppresses the reproduction of bacteria and *Candida albicans* [43].

In this connection, studies in mice conducted by Liuzzi et al. (2005) revealed a crucial role of the proinflammatory cytokine Interleukin (IL)-6 for hypozincemia during the acute phase response [44]. The authors' data indicated IL-6-mediated systemic hypozincemia and zinc accumulation in murine hepatocytes due to an enhanced expression of both, Zip14 and Metallothionein (MT). Whereas Zip14 functions as zinc importer, belonging to the zinc transporter gene family [45], MT serves as small, cytosolic, metal-chelating protein providing zinc binding capacity (see chapter 4). Hence, during inflammation reduction of serum zinc levels is achieved via IL-6-induced upregulation of zinc transporter Zip14 in the liver, thereby promoting zinc uptake from the serum. Concomitantly the expression of zinc chelating molecules is enhanced, leading to sequestration of zinc in the liver. In addition, a recently identified outer membrane receptor in *Neisseria meningitidis* was shown to be involved in zinc acquisition of bacteria [45]. This receptor is upregulated under zinc limited conditions and is thought to control zinc uptake. Homologues of this receptor protein were also found in many other Gram-negative pathogens, particularly in those residing in the respiratory tract [45]. Systemically low zinc levels upon pathogen invasion is therefore a crucial acute phase response in order to mobilize cells of the myeloid lineage, which differentiate to a greater extent during zinc deprivation (as described above), and to keep competition for zinc between pathogen and cells at bay. Yet, a sufficient zinc supply is important for an adequate immune response, as zinc deficient individuals exhibit decreased immune function.

2.1. Zinc deficiency and altered immune function

During the past 40 years, it became evident that zinc deficiency in humans is prevalent and may affect over two billion subjects in the developing world [46-49] (for details see chapter 2). Zinc deficiency occurs on different levels, be it a severe zinc deficiency on the one hand or a marginal zinc deficiency on the other hand. Severe deficiency in zinc may be provoked by the disease *acrodermatitis enteropathica*. Other causes include total parenteral nutrition without zinc, excessive use of alcohol, or following penicillamine therapy. Severe zinc deficiency manifests in bullous pustular dermatitis, alopecia, diarrhea, emotional disorder, weight loss, intercurrent infections due to cell-mediated immune dysorders, hypogonadism in males, neurosensory disorders and problems with healing of ulcers. Severe zinc deficiency is lethal if not detected and reversed in time [46; 50; 51].

Moderate zinc deficiency is caused by nutritional zinc deficiency due to high intake levels of phytate (see chapter 3), which counteracts the absorption of zinc, by malabsorption syndrome, hyperzincuria as seen in liver cirrhosis or sickle cell anemia. Marginal hypozincemia can often be observed in elderly individuals and hemodialysis patients. Growth retardation, male hypogonadism in adolescent, rough skin, poor apatite, mental lethargy, delayed wound healing, cell mediated dysfunctions and abnormal neurosensory changes are possible manifestations of a marginal zinc deficiency. Even mild deficiency of zinc in humans affects immunological function adversely [50; 52; 53].

During the past few years attention has been focused especially on the correlation between the zinc status and the immune function in elderly people. Zinc supplementation in older individuals significantly decreased the incidence of infection [54]. More detailed information about zinc and aging can be found in chapter 16. A causal relationship between zinc deficiency and infections is also demonstrated in a study with severely malnourished Bangladeshi children, where serum and hair zinc was negatively correlated to acute lower respiratory infection [55]. More about the role of zinc in infectious diseases is given in chapter 11. In critically ill patients a decline in serum zinc was observed, which may aggravate the disease and is related to increased mortality [56]. This issue is further discussed in chapter 12. Furthermore, deficiency of zinc enables manifestations of allergies and autoimmune diseases and is the topic of chapter 13 [57-59]. Zinc deficiency is also associated with various types of cancer, for example breast cancer [60], which is a topic in chapter 14. Zinc is required for adequate immune responses, which has been discussed in more detail below.

3. Zinc and the Innate Immunity

Innate or natural immunity provides the first barrier of host defense. Elements of the innate (non-specific) immune system include the anatomical barrier (skin and mucosa), secretory molecules and cellular components. If pathogens successfully cross the epithelial surfaces, they are usually recognized by cellular components of the innate immune system, which induce inflammation in order to eliminate the antigen. Secretory molecules, like the complement system, reinforce and foster the increasing immune response. Zinc molecules have the ability to affect the above mentioned elements of innate immunity.

Maintaining membrane barrier structure and function has been reported to be affected by zinc. Since the intestinal tract is continuously exposed to pathogens and noxious agents, intercellular junctional complexes between neighboring cells are important to create a continuous seal. In a study, zinc deficiency caused alteration in the membrane barrier permeability of endothelial and lung epithelial cells and resulted in gut membrane damage associated with inflammatory cell infiltration; in particular uncontrolled mucosal epithelial transmigration of polymorphonuclear leukocytes (PMNs) was correlated with enhanced development of mucosal inflammation and damage [61]. Consistent with these data, patients with chronic intestinal permeability disturbances are associated with reduced mucosal zinc levels. Moreover, a study by Zhong and coworkers suggested that zinc deprivation due to alcohol exposure disassembled the tight-junction proteins, thereby modulating the epithelial barrier with subsequent increased gut permeability [62]. The importance of zinc regarding the gastrointestinal tract is further discussed in chapter 22.

In addition, secretory molecules also contribute to innate immunity. Apart from signaling molecules -the cytokines- antimicrobial molecules are secreted as well. A zinc-dependent improving effect on mucosal innate immunity was associated with the stimulation of antimicrobial peptide secretion from intestinal epithelium cells [63]. In particular, zinc supplementation induced the production of the antimicrobial peptide LL-37 from Caco-2 cells (human epithelial colorectal adenocarcinoma cell line) in a dose-to-time dependent manner, showing beneficial effects against infectious diseases, particularly diarrhea [63] (see chapter 11). The cathelicidin LL-37 elicits potent antimicrobial activity against a variety of bacteria, including staphylococcal species and *Escherichia coli* as well as fungi, such as *Candida albicans* [64]. A further beneficial aspect of zinc regarding secretory molecules in innate immunity provides its role regarding the bactericidal activity of human peptidoglycan recognition proteins (PGLYRPs). These are secreted innate immunity pattern recognition molecules with zinc-dependent effector function, in most cases against Gram-positive and Gram-negative bacteria [65].

The recruitment of leukocytes from the bloodstream to the site of infection upon pathogen exposure constitutes a significant part of the innate immune response. Leukocytes-direction to the infected tissue involves chemotaxis, adhesion and diapedesis. Chemotaxis allows immune cells to migrate towards the site of injury or infection due to the presence of chemoattractant proteins. At the side of infection, adhesion molecules on the leukocyte-surface bind tightly to complementary receptors expressed on endothelial cells, resulting in adhesion of the leukocyte. Finally, leukocytes transmigrate through the endothelium, enabled by the degradation of the endothelial basement membrane by leukocyte-secreted proteases, a process called diapedesis.

Long-term zinc-deprivation was observed to affect the gene-expression of chemokines,-small chemoattractant proteins- and their receptors, implying that zinc effects chemotactical processes [66]. In addition, zinc deficiency impairs chemotaxis of neutrophils *in vivo* [67]. Moreover, physiologic concentrations of zinc are demonstrated to be necessary for the adhesion of myelomonocytic cells to the endothelium, since cell adhesion was shown to be abrogated during 1,10-phenanthroline (a cation chelator)-induced zinc deprivation [68]. In addition, the human monocytic cell line THP-1 reveals an MCP-1 (macrophage chemoattractant protein-1)-induced increase in intracellular labile zinc concentration, which resulted in triggered monocyte adhesion to activated endothelial cells [69].

The expression of the adhesion molecule ICAM-1 (inter-cellular adhesion molecule-1) that is produced by vascular endothelial cells where it serves as ligand for integrins on activated leukocytes was induced by high zinc doses in human umbilical vein endothelial cells (HUVECs) [70]. Furthermore, zinc concentrations exceeding 500 μM apparently may directly induce chemotaxis of PMNs *in vitro*. This hypothesis is based on the experimental observation that zinc-induced orientation reactions of PMNs resemble polarization reactions induced by the potent chemoattractant peptide N-formylmethionylleucylphenylalanine (fMLP) [71]. Thus, whereas moderate amounts of zinc seem to be required for adequate chemotaxis and cell adhesion, high zinc doses trigger uncontrolled leukocyte infiltration by upregulation of vascular endothelial adhesion molecules and direct induction of chemotaxis, which may result in excessive inflammation.

The cellular components of the innate immunity finally eliminate the antigen and activate the adaptive immune system. The following sections will provide deeper insight into the individual cellular components of the innate immunity, starting with granulocytes, then dealing with monocytes/macrophages, dendritic cells and NK cells.

3.1. Granulocytes

Granulocytes are among the first recruited cells entering infected tissue. They are subdivided into neutrophils, eosinophils, and basophils. Neutrophils, also referred to as polymorphonuclear leukocytes (PMN), are the predominant cells of the innate immune system, whereas eosinophils and basophils constitute only a marginal fraction of granulocytes. The main functions of PMNs are phagocytosis and intracellular killing of invading organisms.

In general, zinc deficiency leads to an aberration in PMN function. In most cases, chemotactic responses were impaired and reversible by zinc supplementation, but the absolute count of PMNs was not affected [72].

Upon phagocytosis, PMNs together with macrophages produce a variety of toxic molecules that participate in intracellular killing of pathogens. Activation of the membrane-bound enzyme NADPH oxidase results in the formation of superoxide radical (O_2^-) by consuming molecular oxygen, describing a phenomenon called oxidative burst. Subsequently, O_2^- undergoes either spontaneous- or enzyme-catalyzed dismutation to hydrogen peroxide (H_2O_2) and further chemical and enzymatic reactions produce a range of toxic chemicals from H_2O_2 , including the hydroxyl radical ($\cdot\text{OH}$) and hypochlorous acid (HOCl). Those reactive oxygen species (ROS) mediate intracellular pathogen killing. Their generation, thereby, is a pivotal protective component of the innate immune system. Transient zinc deficiency may result in a reduced generation of ROS in PMNs as shown during severe lactogenic *acrodermatitis enteropathica* for one infant by Honzik et al. [73].

Even though some experiments suggested an inhibitory effect of zinc on the production of O_2^- [74], several more recent reports support the idea that zinc may stimulate the oxidative burst of PMN [75; 76]. Freitas and coworkers showed that zinc is a potent activator of human PMNs' oxidative burst *in vitro* [77]. Low zinc concentrations (5-12.5 μM) were shown to be capable of activating the NADPH oxidase, mainly via the protein kinase C (PKC), which resulted in the formation of O_2^- . In contrast, higher zinc concentrations (up to 1000 μM) induced a rapid dismutation of O_2^- to H_2O_2 , which in turn is used by myeloperoxidase to generate HOCl [77]. The same study further observed that the use of phosphate-containing incubation medium

abolished the zinc-induced production of ROS due to complex-forming of phosphate and zinc. The activation of NADPH by low zinc concentrations as reported by Freitas et al. [77] are in contrast to the inhibitory zinc effect on the production of O_2^- as suggested by Henderson et al., [74], but might be explained by the use of different buffers.

PMNs of elderly individuals display impaired chemotaxis, phagocytosis, and oxidative burst with subsequent less ability to destroy the pathogens [78; 79]. In addition, PMNs of elderly show a decreased rescue from apoptosis after activation based on a lack of responsiveness to granulocyte macrophage colony-stimulating factor (GM-CSF), which results in a blunted PMN response to infections [80]. Furthermore, the enzyme superoxide dismutase (SOD), which is necessary in avoiding ROS produced by oxidative burst, is limited in elderly subject [78], since SOD requires zinc for proper function [81]. High amounts of ROS are related to induction of inflammation [82]. Consequently, aging, which is often associated with zinc deficiency, limits the protection to infection due to impaired PMN function and triggers chronic inflammation.

Interestingly, the activation of human PMNs was recently demonstrated to be modulated by zinc in association with hydroxyapatite (HA) induced inflammation. HA is widely used in coating biomaterials and interaction of HA with PMNs leads to PMN activation and subsequent expression of the chemokine IL-8 causing inflammation. Substitution of 5%HA with zinc opposes the effect of HA alone, indicated by decreased expression of IL-8 and other inflammatory mediators [83].

To conclude, zinc deficiency may lead to prolonged impairment of PMN cell function and increased chronic inflammation, while zinc supplementation in small concentrations seems to boost PMN activity. A sufficient amount of zinc is hence required to maintain the first line of defense.

3.2. Monocytes/Macrophages

As PMNs, monocyte/macrophages belong to the group of phagocytic cells. However, in addition to phagocytosis and killing of pathogens, upon preactivation monocytes/macrophages present antigens to T cells of the adaptive immune system, thereby acting as so-called professional antigen presenting cells (APCs).

In order to recognize certain components of pathogens, known as pathogen associated molecular patterns (PAMPs), a wide repertoire of specific receptors is expressed on the surface of monocytes, e.g. toll-like receptors (TLR). For example, TLR-4 becomes activated by the endotoxin lipopolysaccharide (LPS), a component of the cell wall in Gram-negative bacteria. Activation of TLR-4 in turn induces several signaling pathways, including mitogen-activated protein kinases (MAPK), protein kinase C (PKC), phospho-inositide 3 kinase (PI3K), Src family tyrosine kinase and NF κ B, leading to the expression and release of several proinflammatory cytokines such as IL-1 β , IL-6 and tumor necrosis factor (TNF)- α [84].

Notably, metallothionein (MT)-KO mice-derived macrophages display defects of phagocytosis, antigen presentation and cytokine production, thereby indicating an important role of MT in macrophage function [85]. Since MT is strictly associated with zinc homeostasis (see chapter 4), one can assume that the latter is an important player in macrophage function. Indeed, LPS-induced stimulation of human monocytes led to increased free intracellular zinc concentration in less than two minutes [86]. This zinc signal is required for LPS-induced signal transduction, since zinc chelation abrogated

LPS-induced transcription and release of proinflammatory cytokines [86; 87]. Signaling events that involve free intracellular zinc are not restricted to monocytes or cells of the immune system. The role of zinc as a signaling modulator is further discussed in chapter 6.

Interestingly, resting macrophages infected with the intracellular fungal pathogen *Histoplasma capsulatum* effectively suppress yeast replication when stimulated with the cytokine GM-CSF [84]. On the other hand, the infected macrophages fail to kill the pathogen when stimulated with IL-4. Whereas GM-CSF is associated with decreased intracellular zinc concentrations, IL-4 was observed to enhance the intracellular zinc level. This leads to the conclusion that intracellular zinc deprivation may exert an effective host defense mechanism utilized by macrophages against fungal pathogens which is supported by differentiation of cells of the myeloid lineage triggered by hypozincemia.

The Human Immunodeficiency Virus (HIV) causes acquired immunodeficiency syndrome (AIDS) by infecting vital immune cells such as monocytes, dendritic cells and T helper cells resulting in apoptosis of affected immune cells. Depletion of the cell-mediated immunity enhances the susceptibility towards pathogens. Interestingly, different immune cell types are not equally prone to HIV-induced apoptosis. For example, CD4⁺ T cells, being part of the adaptive immunity, are the main cell subset to be depleted during chronic HIV infection via various mechanisms, including activation-induced apoptosis. CD4⁺ T cells have a lower intracellular zinc level than monocytes, which seem to resist apoptosis [88; 89]. Consistently, circulating monocytes of HIV patients show higher intracellular zinc levels than lymphocytes, due to an increased expression of MT family genes [90]. It has been hypothesized that differences in intracellular zinc levels may be responsible for the differential response of various cell types to apoptosis [91; 92] (see chapter 5). Higher intracellular zinc levels may provide better resistance to apoptosis during HIV viremia [90].

Recognition of pathogens via PRR in monocytes/macrophages does not only induce cytokine production but also initiates phagocytosis of foreign antigens. During phagocytosis, macrophages rearrange their cytoskeleton structure and engulf the particle with so-called pseudopods. Once ingested, the antigen becomes digested in the phago-lysosome and presented to lymphocytes via MHC molecules. Phagocytosis is affected by zinc since macrophages derived from zinc treated children suffering from enterotoxigenic *E.coli*-induced diarrhea show improved phagocytic activity [93]. The observation that zinc increases the phagocytic capacity of canine peripheral blood phagocytes *in vitro* accords well to these results. [94]. Peripheral blood monocyte-rich cells showed increased phagocytosis after zinc supplementation. Additionally, in the same study, incubation of PBMC with zinc induced TNF- α secretion into the culture supernatant, which in turn enhanced phagocytic capacity of canine peripheral blood phagocytes such as PMN and monocyte-rich cells [94]. Hence, zinc might directly induce phagocytic capacity in macrophages, whereas the enhancing effect in PMNs may be rather indirect, caused by zinc-induced TNF- α production in PBMCs.

Besides phagocytosis, the expression of monokines (monocyte-derived cytokines) is influenced by zinc in a concentration-dependent manner. Low zinc concentrations stimulate the release of IL-1 β , IL-6 and TNF- α in PBMCs. This effect is related to the monocyte fraction since the production of these cytokines is maintained in the absence of T cells [95]. TNF- α mRNA is shown to be directly induced by zinc [96]. In contrast, higher zinc concentrations negatively regulate TNF- α , IL-8 and IL-1 β gene expression, as demonstrated in the monocyte/macrophage cell line HL-60 [97]. These results

demonstrate that low zinc concentrations interact synergistically with LPS to produce higher amounts of inflammatory cytokines. Whereas low zinc levels act rather proinflammatory, higher zinc concentrations repress monokine production [98]. The mechanism responsible for the zinc-related suppression of monokine production is a zinc-induced inhibition of the phosphodiesterase (PDE), leading to elevated levels of the second messenger cGMP which is followed by a subsequent suppression of the NF κ B-dependent TNF- α and IL1- β expression [99; 100].

Deprivation of zinc negatively affects monocyte function *in vivo*, especially the production of an adequate cytokine release following infection. In a study involving elderly subjects, adjustment of the intracellular zinc level by moderate supplementation reduced basal cytokine release [101]. Especially IL-6 production was strongly diminished after zinc treatment, followed by reduced spontaneous inflammatory activity and ameliorated termination of inflammatory responses. In contrast, IL-6 production after LPS stimulation *ex vivo* was increased in zinc supplemented probands, which is consistent with the finding that LPS signaling requires zinc signals for adequate signaling [86]. In accordance with this, zinc supplementation in elderly subjects increases the plasma zinc concentration associated with concomitantly decreasing plasma IL-6 levels [102]. Furthermore, cells of the human monocytic leukemia cell line THP-1 showed zinc-induced decreased levels of TNF- α , IL-1 β and reduced NF κ B-activity compared with zinc deficient cells [102]. This supports the observation that zinc deficiency provokes spontaneous cytokine release which increases the risk of chronic inflammation, whereas restoring of the zinc level counteracts inflammatory cytokine production. Notably, a number of other genes with a functional relationship to TNF are also affected by altered zinc status, showing that the effect of zinc status on pro-inflammatory signaling is not an isolated event for a few cytokines, but involves many genes [103].

Taken together, zinc affects the activity of monocytes/macrophages in a manifold way on different crossing levels. Zinc is involved in monocyte/macrophage development, as discussed earlier and functional activities, particularly in phagocytosis and proinflammatory cytokine production.

3.3. Dendritic cells

Dendritic cells (DC) represent the third class of phagocytic immune cells. They are clustered at body gateways and transport antigens from the periphery to lymphoid tissue. Therefore they are likewise named “sentinels” of immunity. DCs perform function as professional APCs, expressing between ten and hundred times more antigen-presenting MHC molecules than other APCs, and are able to activate antigen-specific T lymphocytes, the key players of adaptive immunity. Due to this property, DCs form a crucial link between the innate and the adaptive immune system. In addition to their immunostimulatory function, DCs also play a pivotal role in maintaining peripheral tolerance by regulating T effector cell activity and by inducing regulatory T lymphocytes (Tregs), T cells with immunosuppressive functions [104]. In order to recognize pathogens DCs express pattern recognition receptors (PRRs) such as TLRs. Stimulation of DCs via TLR induces the activation of immature cells, indicated by upregulated MHC molecules and co-stimulatory molecules CD40, CD80 and CD86. Furthermore, TLR-stimulation causes proinflammatory cytokine production due to activation of several transcription factors, e.g. NF κ B [105]. Experiments showed that TLR-4 activation by LPS alters the expression of zinc transporters in DCs, among them

the zinc importer Zip6. Subsequent downregulation of Zip6-expression results in a net decrease of intracellular zinc [106]. Consistently, TPEN-induced zinc chelation triggers DC maturation as reflected by increased MHC expression, thereby mimicking the LPS-induced DC activation. However, CD40 and CD80 expression remains constant after zinc removal, implying that zinc acts in a very specific manner. In contrast, zinc supplementation and overexpression of Zip6 inhibit the LPS-induced upregulation of MHC- and costimulatory molecules.

Two molecular mechanisms were suggested for the divergent effects of zinc. The first mechanism assumes that zinc deprivation inhibits the endocytic removal of MHC from the plasma membrane, causing cell surface MHC-molecule accumulation. The second mechanism suggests that high zinc concentrations inhibit the LPS-induced migration of MHC-containing vesicles to the plasma membrane [106; 107].

Interestingly, an increase in the expression of the zinc finger protein A20 was associated with activated DCs [108]. A20 is a zinc finger protein with ubiquitin-modifying activity and has been described as a negative regulator of TNF receptor and TLR family signaling in a variety of cell types [109]. The activation of DCs via TLR3 signaling pathway resulted in increased A20 protein levels. Therefore, A20 protein can be considered to act as a brake during TLR activation, since down-regulation of A20 enhances the functional activation of DC, shown in increased IL-6 production [108]. The induction of A20 mRNA as well as the generation of the A20 protein was demonstrated to be zinc-dependent in premonocytic, endothelial and cancer cells [110]. Maybe downregulation of the intracellular zinc level subsequent to TLR activation is a mechanism to modulate and fine-tune the immune response of DCs. High expression of A20 may inhibit the production of costimulatory molecules and proinflammatory cytokines, whereas a low A20 protein level may result in spontaneous and enhanced immune response. This is consistent with abrogated DC maturation in response to TLR activation due to zinc supplementation (hence enhanced A20 expression), whereas zinc deficiency (abrogated A20 production) triggers DC maturation. In addition, A20 silenced hyperactive DCs have divergent effects on the T cell populations: inhibition of Tregs and hyperactivation of T effector cells [111]. Thus, the reduction of cellular zinc in DCs presumably allows for a negative regulation of DC activation, while maintaining the stimulatory capacity of DCs. Zinc might therefore serve as a fine tuner of DC activity and as a modulator of the T cell activation.

Moreover, zinc is associated with the formation of ceramide in different cell types, which is known to trigger suicidal cell death. Lately, studies with murine bone-marrow derived DCs (BMDCs) found that treatment with low zinc concentrations stimulated the formation of ceramide and subsequently induced suicidal cell death in DCs [112]. The importance of ceramide formation for zinc-induced apoptosis was demonstrated using acid-sphingomyelinase KO mice-derived BMDCs, which are unable to produce ceramide. Those cells resisted zinc-induced apoptosis. It has to be pointed out that only apoptotic cell death induced by zinc concentrations up to 100 µM were ceramide-dependent, whereas higher zinc concentrations increased apoptotic death in wild-type and KO-mice. One can speculate that by lowering the intracellular zinc level LPS fosters the survival of DCs. Indeed, the data indicate an anti-apoptotic effect of LPS on DCs, but this effect is not believed to be dependent on acid sphingomyelinase-activation since ceramide formation was not significantly altered by LPS-signaling. Nevertheless, the influence of zinc on DC survival may modulate the course of inflammation and thus infections.

In summary, DC activity is strictly regulated by zinc homeostasis. Zinc deficiency enhances and dysregulates DC activation, resulting in an overproduction of proinflammatory cytokines. High zinc concentration, on the other hand, dampens DC activity.

3.4. NK cells

NK cells are a subset of lymphocyte which account for approximately 15% of human peripheral blood lymphocytes. Their main function is the killing of infected cells as well as tumor cells by triggering apoptosis. In contrast to B and T cells, the other lymphocyte subsets, NK cells do not express antigen-specific receptors or develop memory cells and are therefore considered to be part of the innate immune system. For the recognition of virus-infected or malignantly transformed cells, NK cells detect molecular alterations on cell surfaces, particularly the reduced expression of MHC I molecules. For this purpose, NK cells bear MHC I molecules-ligands on their surface, the killer inhibitory receptors (KIR), detecting the level of MHC I expression of the target cell. In the case of diminished MHC I expression NK cells become activated due to a lack of KIR-engagement, which initiates cytotoxic mechanisms. In addition to inhibitory molecules, such as KIR, NK cells also express a wide variety of invariant activating receptors, for example the Fc receptor for IgG, enabling interaction with antibodies specifically bound to antigens to induce the killing. This process is called antibody-dependent cellular cytotoxicity (ADCC).

NK cells depend in their activity and number on the serum zinc level, which is reflected by impaired NK cell function during zinc deficiency [113]. In the case of MHC molecule recognition by KIR receptors, zinc has been proven to be a necessary element due to its contribution to NK cell p58 killer cell inhibitory receptor interaction with MHC I molecules (HLA-C) on target cells [114]. Stimulatory signals, on the other hand, are not zinc-dependent [114]. As the inhibition of the killer function of NK cells depends on zinc, zinc deficiency can be considered to cause unspecific killing by NK cells. However, the lytic activity of NK cells is impaired during zinc deficiency and can thereby counteract the unspecific killing [115; 116]. Interestingly, impaired cytotoxic activity on a cell-cell basis as well as reduced proliferation in response to IL-2 in NK cells of elderly individuals is compensated by an increased total number of NK cells and their increased percentage among circulating cells [117; 118].

In contrast to this finding, a study by Muzzioli and coworkers revealed that proliferation and differentiation of CD34⁺ progenitor cells towards NK cells was impaired in older people which was partially corrected by zinc supplementation [119]. In this study, the effect of physiological zinc concentrations on the kinetic of the differentiation of CD34⁺ cell progenitors towards NK cell was investigated *in vitro*, comparing young and old healthy donors. Under the same experimental conditions, the number of cells, their phenotype and cytotoxicity, and the kinetics of differentiation of CD34⁺ cell progenitors during *in vitro* culture were different in old and young subjects, revealing a lower cell number in aging subjects. In addition, zinc played a crucial role in NK cell development by significantly increasing the number and cytotoxic activity of NK cells in young and old donors, implying that zinc is able to partially correct impaired proliferation and differentiation of CD34⁺ progenitor cells towards NK cells. A zinc-induced modulation of the GATA-3 transcription factor, one of the most important transcription factors for NK cell maturation and activation, was shown to be associated to the above described effects as zinc increased the expression of GATA-3

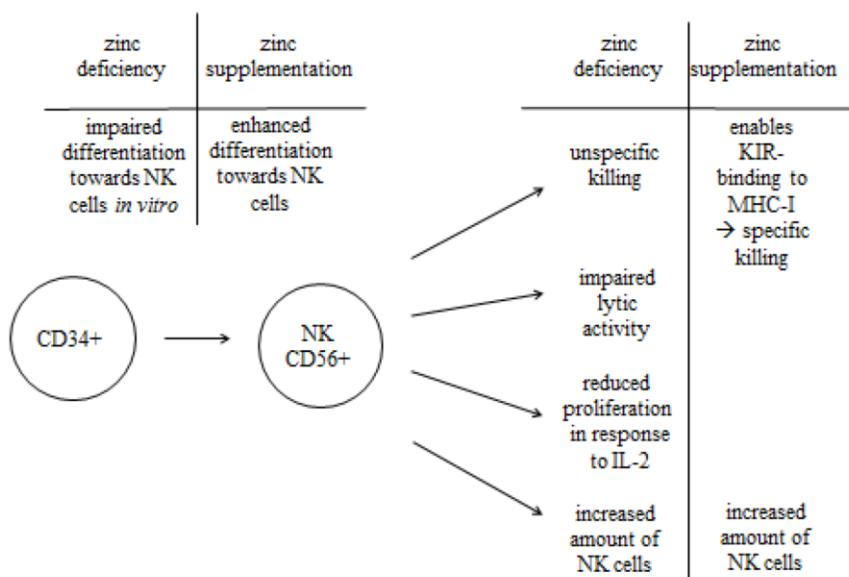


Figure 1. Effect of zinc status on NK cell immunity. Differentiation, proliferation, and function of NK cells are affected by zinc status. Zinc deficiency impairs the differentiation of CD34⁺ progenitor cells into NK cells *in vitro*, causes reduced proliferation in response to IL-2, which is counteracted by an increased amount of NK cells. Zinc deficiency induces unspecific killing of NK cells, but the lytic activity is downregulated. Zinc supplementation enhances the differentiation towards NK cells, increases the amount of NK cells and is required for proper KIR-binding to MHC-I molecules on target cells.

mRNA, showing higher peaks in old than in young donors [119]. These results are, on the one hand, consistent with the aforementioned finding that zinc deficiency reduces the lytic potential of NK cells, but on the other hand, stand in contrast to the finding of increased NK cell number in zinc-deficient elderly. It has to be taken into account that the study, which investigated the differentiation of NK cells from CD34⁺ progenitor cells, was exhibited *in vitro* and did not analyze the number of peripheral NK cells of the old and the young subjects.

A close relationship between the quantity of NK cells and zinc is furthermore shown in the finding that *in vitro* zinc supplementation of PBMC amplified the amount of CD56⁺ NK cells, accompanied by enhanced interferon (IFN)- γ production [120]. This is consistent with the fact that zinc supplementation of CD34⁺ progenitor cells of young, zinc adequate subjects showed enhanced differentiation towards NK cells.

In general, higher incidences of cancer and viral infections are associated with zinc deficient elderly and may be due to impaired NK cell function. In summary, zinc is involved in NK cell development, maturation, and function which reveal the essentiality of zinc regarding virus protection and cancer prevention. An overview about the effect of zinc status on NK cell differentiation and function is presented in figure 1.

Taken together, the innate immunity is highly affected by fluctuations in zinc homeostasis. Naturally occurring zinc deficiency as observed during the acute phase response enhances the proliferation of PMN, monocytes, and DC, thereby triggering the innate immune response. Long term zinc deficiency, on the other hand, causes

dysregulated innate immune responses, shown by impaired immunity against pathogens concomitant with severe inflammation and enhanced susceptibility towards cancer development. Increased bacterial burden, systemic inflammation, vital organ damage, and mortality can occur after infection in zinc deficient individuals as demonstrated in a zinc deficient mice-based small animal model of sepsis [102]. Zinc supplementation prior to infection reduces the symptoms. Therefore, zinc is considered to exert anti-inflammatory potential. In addition to innate immunity, cells of the adaptive immune system are also responsive to alterations in zinc levels which is topic of the next section.

4. Zinc and the Adaptive Immune System

The adaptive immune system includes the two main subsets of lymphocytes, B and T lymphocytes, which, in contrast to the cells of the innate immune system, carry specific receptors to recognize a certain target structure and are able to establish immunological memory.

The development of immunological memory allows a more rapid and efficient immune reaction when the same antigen is encountered which provides long-lasting protection against it. After specific antigen recognition, clonal expansion of a small subset of selectively activated lymphocytes initiates the subsequent adaptive immune response. Activated B cells develop into Ig-secreting plasma cells, thereby contributing to the humoral immunity. Activated T cells, on the other hand, exert cytotoxic effects and helper functions being part of the cell-mediated immunity. Altered zinc homeostasis affects both, humoral and cell-mediated immunity. As an example for the importance of zinc in the adaptive immune system, naive and memory T and B cell responses upon vaccination are declined during zinc deprivation [82; 121].

4.1. T cells

T lymphocytes exert effects on other cells, either by regulating the activity of immune cells or by killing cells that are infected or transformed. Based on their function mature T cells can be divided into different subgroups; CD8⁺ cytotoxic T lymphocytes (CTLs) mediate cell death via cell-cell interaction and CD4⁺ T helper cells (Th) promote other immune cells to exhibit immune functions. Cells of the Th subset can be further subdivided into Th1, Th2, Th17 and regulatory T cells (Tregs). Th1 cell-secreted cytokines, such as IFN- γ and IL-2, are mainly involved in activating macrophages (cell-mediated responses), while Th2 cells are generally known to promote antibody production by stimulating B cells via IL-4 (humoral responses). Th17 cells produce several pro-inflammatory cytokines, particularly IL-17A, whereas Tregs suppress the activity of autoreactive, hypersensitive, and allogenic T cells.

The prominent role of zinc in T lymphocytes becomes visible in the impact of zinc deprivation on T cell immunity, comprising T cell development as well as the polarization into effector cells and the functional capacity of T cells [122]. A general effect of zinc deficiency including all T cell subsets is a diminished proliferative response after mitogen-stimulation [97; 116; 123].

Lymphopoiesis is, as described in detail above, highly zinc-dependent. Reduced zinc-availability causes thymic atrophy, impaired T cell development and decreased T

cell count [14; 124; 125]. In an experimental model of zinc deficiency the recruitment of naive T helper cells and the percentage of precursor cells of CTLs were both diminished [126]. The hormone thymulin, a nonapeptide secreted by thymic epithelial cells, requires zinc as a cofactor to achieve its active form. Thus, the zinc-free form of thymulin is functionally inactive. In its biologically active form this hormone is essential for T cell differentiation and T cell function, and zinc deficient mice showed diminished levels of biologically active thymulin in the circulation [127]. *In vitro* supplementation of serum zinc restored thymulin-activity, emphasizing a direct effect of serum zinc on thymulin activity [128]. Additionally, reduced thymulin activity was observed in moderately zinc deficient humans and zinc supplementation *in vitro* and *in vivo* provoked thymulin-activity reconstitution [129].

In children suffering enterotoxigenic *E. coli*-induced diarrhea zinc treatment improved the naive/memory T cell ratio [93]. This finding affirms the necessity of zinc for the generation of naive T cells. Accordingly, aging as a model for chronic mild zinc deficiency is associated with a decreased ratio of CD4⁺CD45RA⁺ (newly produced, naive) to CD4⁺CD45R0⁺ (memory) cells [130]. More information about aging and zinc is provided in chapter 16.

Furthermore, zinc influences the polarization of naive T cells into the different subgroups. Zinc deficiency is associated with a decreased ratio of CD4⁺ to CD8⁺ cells [129]. In addition, the Th1/Th2 balance gets disturbed during zinc deprivation, leading to a reduced production of the Th1 cytokines IFN- γ and IL-2. Whereas the cell-mediated response is disrupted, the humoral response remains less affected, indicated by an unaltered production of IL-4 and IL-10 [131-133]. According to these findings, the delayed type hypersensitivity (DTH), a mainly Th1 mediated hypersensitivity reaction, failed in mice during zinc deficiency [134]. Zinc supplementation is able to restore Th1 mediated responses indicated by normal cytokine profiles, improved DTH -reaction and lymphocyte proliferation. Imbalanced Th1/Th2 ratio was also observed in young subjects during experimentally caused zinc deficiency [53]. Despite unaltered plasma zinc levels, these probands showed significant effects on the production of IFN- γ , IL-2, and TNF- α . Notably, a reduction of plasma zinc levels towards a concentration that affects the immune system can remain scarcely within the reference values [135]. More evidence for altered Th1/Th2 cytokine balance is provided in elderly, marginally zinc deficient individuals that show higher IFN- γ production after zinc supplementation in response to T cell stimulation [101]. A study dealing with oral inactivated cholera vaccination in young children in a cholera endemic country revealed an enhancing effect of zinc supplementation on the Th1 response, particularly in formerly zinc-deficient children [136].

Even though one recent study reports somewhat contradictory results, namely that zinc deficiency was associated with increased IFN- γ production upon *ex vivo* stimulation of PBMC with *Plasmodium falciparum* parasitized erythrocytes (pRBCs) [137], the majority of studies still reveal an impaired Th1 response during zinc deprivation.

The physiological consequences of zinc-enhanced Th1 response become apparent in rats infected with the parasite *Trypanosoma cruzi* [138]. During the acute phase of infection, supplementation with zinc triggered the immune reaction, as shown by increasing concentrations of the Th1 cytokine IFN- γ accompanied by elevated macrophage counts, accelerated concentrations of nitric oxide (NO), and significantly reduced parasite load. Th1 lymphocytes are generally known to promote cellular immunity against intracellular pathogens and are therefore associated with a partially

protective immunity against the intracellular parasite *T. cruzi* [139]. While a predominant Th1 response supports resistance towards *T. cruzi*, Th2 immune response is associated with increased susceptibility to the infection [140; 141]. Hence, the zinc-induced shift towards Th1 immune response, indicated by increased IFN- γ production, correlated with enhanced resistance to acute infection with *T. cruzi* [142]. Furthermore, Th1 cell-dependent IFN- γ production is pivotal for the activation of macrophages, which in turn are the ultimate host cells for a number of intracellular pathogens [143]. As seen in the significantly enhanced number of macrophages in zinc supplemented rats infected with *T. cruzi*, zinc deficiency and subsequent abrogation of Th1 immune response indirectly affect the immunogenic capacity of macrophages. Moreover, zinc supplementation during pregnancy in mice infected with the Y strain of *T. cruzi* provided reduced parasite burden in the placenta, implying that zinc supplementation may attenuate the infection of the fetus by ameliorating the Th1 response [144].

To further study the effect of zinc on the production of Th1 cytokines, the expression of IL-2 in PHA/PMA-activated HUT-78 cells, cells of the human Th0 malignant lymphoblastoid cell line, was examined. In zinc deficient HUT-78 cells, the gene expression of IL-2 was decreased by 50% compared with that in zinc sufficient cells [131]. A significant effect of zinc was also shown on the gene expression of IL-2 receptors α and β . Binding of NF κ B to DNA was decreased in zinc deficient cells. The binding of recombinant NF κ B (p50)₂ to DNA was demonstrated to be zinc-specific [145]. NF κ B binds to the promoter enhancer area of IL-2 and IL-2 receptor α genes. Therefore, the adverse effect on NF κ B-binding to DNA causes decreased gene expression of IL-2 and the IL-2 receptor in zinc deficient HUT-78 cells.

Further aspects with regard to zinc-dependent Th1 cytokine production are provided by studies with activated primary T cells. In detail, TCR-mediated activation of primary T cells from human subjects via microbeads conjugated to antibodies against human CD2, CD3, and CD28 resulted in IFN- γ expression, which accelerated significantly when oral zinc supplementation was provided to the subjects [146]. Additionally, the zinc transporter ZIP8 was demonstrated to be highly upregulated during T cell activation. Its importance in T cell activation was supported by the finding that siRNA-mediated suppression of hZIP8 expression decreased the IFN- γ production of activated T cells, whereas ZIP8-overexpression resulted in enhanced IFN- γ production [147]. ZIP8 localizes to lysosomes, where it mediates zinc influx into the cytosol. The results of the study by Aydemir et al. suggest a ZIP8-dependent influence on T cell activation through a change in the cytoplasmic zinc concentrations. This finding assigns a role to zinc as a signaling molecule involved in T cell activation suggesting that increasing cytosolic zinc inhibits calcineurin (CN) activity, leading to activation of the transcriptionfactor CREB and subsequently to the expression of IFN- γ . Recently, Yu et al. reinforced the idea of zinc acting as an ionic signaling molecule after T cell activation [148]. They measured increased cytoplasmic zinc concentrations in CD4 $^{+}$ T cells from peripheral blood within 1 minute after TCR triggering induced by mDCs loaded with superantigen. Silencing of ZIP6, a cell membrane zinc transporter, inhibited the zinc influx. As a result from the inflowing zinc ions the TCR signaling was shown to be calibrated inasmuch as ZAP-70 phosphorylation was enhanced, SHP-1 recruitment to the TCR-activation complex was reduced, and calcium influx was unaltered. Thereby, suboptimal stimuli suffice to induce a T cell response provided that the extracellular zinc bioavailability increases.

Further details in terms of zinc signaling are discussed in chapter 6. Notably, the induction of IFN- γ production by zinc supplementation links the zinc status to Th1/Th2 polarization and matches the observations that zinc deficiency leads to reduced Th1 immune response.

In contrast, Hayashi and coworkers (2008) observed a zinc-dependent inhibition of IFN- γ expression in activated Jurkat cells (human T cell line) by using a higher zinc concentration. The authors claim that exogenous zinc attenuates IFN- γ mRNA expression by downregulation of the calcium-independent PKC-AP-1 signaling pathway [149]. However, it has to be taken into account that the experimental settings differed from the study of Aydemir et al. in that Hayashi et al. (2008) using varying zinc concentrations and stimulating the cells for different time periods. Moreover, both groups worked with different cells, primary cells on the one hand and a cell line on the other, which may also contribute to the different results.

Interestingly, a zinc concentration-dependent inhibition of IFN- γ production in stimulated T cells was also detected in the study of Aydemir et al., who observed a biphasic effect of zinc on IFN- γ production with a maximum peak at a concentration of 3.1 μ M zinc supplement and a diminished IFN- γ expression at 25 μ M of zinc, suggesting that additional zinc in low concentrations positively affects T cell activation. In contrast, zinc concentrations higher than physiological levels rather suppress T cell activation, notably, without affecting cell viability. This divergent concentration-dependent effect of zinc on IFN- γ production is also underlined by the fact that oral zinc supplementation of human subjects with 15 mg zinc/day increased the production of IFN- γ by PBMCs after *ex vivo* activation [146], whereas zinc supplementation of adult human subjects with 80 mg zinc/day showed suppressed IFN- γ secretion when activated in a mixed lymphocyte culture (MLC) *ex vivo* [150]. The idea is further supported by the finding that the MLC dependent IFN- γ production is suppressed in the presence of 50 μ M exogenously added zinc *in vitro* [151]. Zinc levels below 50 μ M were not able to suppress IFN- γ secretion significantly. This matches the results of Hayashi and coworkers, who likewise present an inhibitory effect of zinc on the PMA/PHA activated Jurkats for zinc concentration of 50 μ M and higher. In accordance, supplementation with high zinc concentrations (100 μ M) suppresses IL-1 β -stimulated IFN- γ production in primary human T cells and inhibits IL-1 β -dependent proliferation of cells from a murine T cell line. This phenomenon is mediated by zinc-induced inhibition of the IL-1 type I receptor associated kinase IRAK, which was successfully verified by immunoprecipitation [152; 153].

The mechanisms responsible for the enhanced IFN- γ production upon T cell stimulation at lower zinc concentrations and the suppression of IFN- γ production by high zinc concentrations need further elucidation. Most likely, altering plasma zinc concentrations have varying effects on different signaling pathways. Another possible explanation may be that zinc in higher levels induces the Th cell polarization in favour of regulatory T cells (Rosenkranz et al. unpublished data). Since Tregs exert suppressive functions on activated Th1 cells [154] decreased IFN- γ production by Th1 cells may be due to enhanced Treg activation. In any case, the hampering effect of higher zinc concentrations on the MLC may be relevant regarding the allogeneic immune reaction following transplantations.

Not only the polarization into Th1 or Th2 cells can be modified by zinc, but lately inhibition of Th17 cell development was also related to zinc ions [59]. Th17 cells produce several pro-inflammatory cytokines, particularly IL-17A, and play a critical role in the pathogenesis of autoimmune diseases [155; 156]. Their development is

Table 1. Effect of zinc deficiency on immune functions in experimental human models

Variables
1. Thymulin activity decreased; corrected by both in vivo and in vitro zinc supplementation
2. T cell subpopulation studies CD4+ to CD8+ Ratio decreased CD4+ CD45RA+ to CD4+ CD45R0+ Borderline significant decrease
3. Th1 cytokines decreased IL-2 IFN- γ
4. Th2 cytokines did not change IL-4, IL-10
5. NK cell lytic activity decreased

controlled by IL-6-induced STAT3 activation [157]. In this experimental setting, mice were injected with type II collagen, the major structural component of connective tissue, resulting in the development of Th17-mediated collagen induced arthritis (CIA), which is known to depend on the pro-inflammatory cytokines IL-6 and IL-17A [158; 159].

The addition of 3000 ppm zinc into the drinking water inhibited CIA development in mice, concomitant with decreased serum levels of IL-17A and reduced Th17 cell counts. These effects were shown to be directly associated with zinc-induced suppression of STAT3, a signaltransduction molecule that is crucial for Th17 cell development. Since IFN- γ whose production can be triggered by zinc supplementation negatively regulates the development of Th17 cells [160], the zinc-induced amelioration of CIA development might be based on an increased IFN- γ production. However, whereas Th17 cell development was demonstrated to be inhibited by zinc, the Th1 immune response remained unaltered [59]. Hence, anti-inflammatory effects of zinc may also be obtained by suppression of Th17 activity via STAT3 inhibition [59]. Yet, it should be considered that in this experimental setting mice were treated with high zinc dosages which may evoke zinc toxicological effects responsible for the observed zinc effects.

Taken together, evidence supports that the polarization of Th cells is modulated to a certain degree by the zinc level, thereby affecting the immune system in manifold ways. Table 1 summarizes the effect of zinc deficiency on T cell function found in experimental human models. Th1 cells are known to promote macrophage activation and production of complement fixing and opsonizing antibodies. IFN- γ is the major component of the Th1 response panel and upregulates MHC I antigen expression. Decreased Th1 immunity during zinc deficiency therefore causes impaired cell-mediated immunity. Additionally, a concomitant shift towards Th2 immunity increases the likeliness of allergic immune responses and enhanced Th17 development promotes the development of autoimmune diseases during zinc deficiency due to limited suppression of Th17 development. Additionally, the fact that Tregs strongly rely on the Th1 cytokine IL-2 to exert immune suppressing function [161] may also contribute to enhanced undesirable immune reactions during zinc deficiency. The connection between zinc and infectious diseases, autoimmune diseases and allergies will be discussed in more detail in chapters 11 and 13.

Although the ratio of CD4⁺ (Th) to CD8⁺ (CTL) decreases upon zinc deficiency, the development of cytotoxic precursor T cells as well as the function of peripheral CTLs is impaired under that condition [162]. This is shown by the fact that *in vivo* generated cytotoxic T killer activity to allogeneic tumor cells is abrogated in zinc deficient mice. Additionally, an impaired cell-mediated response to allogeneic tumor cells in zinc deficient mice was reported [163-165]. Studies in experimental human model showed that the percentage of CD8⁺CD73⁺ T cells is decreased during zinc deprivation which can be corrected by zinc supplementation [53]. CD8⁺CD73⁺ lymphocytes are predominantly precursors of CTL, and the presence of CD73 molecule on CTL is required for antigen recognition, the proliferative process, and for generation of the cytolytic process.

Reduced CTL proliferation and activation during zinc deficiency are likely to be associated with abrogated IL-2 production, because IL-2 serves as an early factor for clonal expansion and differentiation of effector function of antigen reactive CTLs as well as Th cells. Moreover, IL-2 dependent proliferation of T cells was recently demonstrated to be promoted via zinc signals [166]. The murine cytotoxic T cell line CTLL-2 and primary human T cells revealed increased free cytoplasmic zinc levels upon IL-2 receptor stimulation which apparently is required for IL-2 induced ERK signaling and subsequent T cell proliferation. Thus, zinc is not only a prerequisite for adequate Th1-dependent IL-2 production, but likewise substantial for functional IL-2 signaling (see chapter 6).

Another way of zinc to interfere in T cell activity is the modulation of T cell recruitment. Naive lymphocytes circulate in the blood stream and enter the lymphnodes where they can be activated by specific APCs. Activation leads to proliferation and differentiation into effector cells, which migrate to the site of infection to exert immunological functions. The recruitment of lymphocytes involves several cell adhesion molecules, e.g. CD44. Although a variety of cells express CD44, it is particularly involved in T cell spreading [167]. CD44-induced spreading requires the activation of Src family kinases [168] for actin-rearrangement, a crucial step for cell migration [169]. The association of CD44 and Lck (a member of the Src family kinases) depends on zinc, as chelation of zinc by the divalent metal ion chelator 1,10-phenanthroline abrogated CD44 mediated T cell spreading [170]. Whereas one can assume that zinc deficiency might therefore impair T cell spreading, upregulation of Lck in T cells was observed during zinc deficiency, maybe compensating the diminished availability of zinc ions [171; 172]. Completely abrogated zinc levels, on the other hand, as induced by zinc chelators, prevent the association of Lck and CD44 causing inhibited T cell spreading, which furthermore highlights the role of zinc in efficient T cell function.

Besides facilitating T cell migration Lck molecules have multiple functions in T cells. Especially they are involved in TCR signal transduction by associating with CD4 and to a lesser extent with CD8. The association between Lck and each co-receptor is coordinated by zinc ions, based on high affinity binding to the cysteine motifs, connecting both proteins [173; 174]. A second zinc-dependent interface site is required for homodimerization of Lck in order to promote its full activation. This interaction proceeds in the physiologically relevant concentration range of free zinc [175].

The multiple effects of altered zinc homeostasis on T cell function and T cell-mediated immune response are striking. They involve modulation of T cell development, polarization into effector cells, and functional effectiveness. Disturbances in zinc homeostasis affect not only T cell-dependent immunity but also B cell-function,

though surprisingly to a much lesser extent. This phenomenon will be further discussed in the next section.

4.2. B cells and Antibody Production

The B cell population constitutes 5-15% of human blood lymphocytes. Their main function is the secretion of soluble recognition molecules called immunoglobulins (Ig) or antibodies. These bind specifically to a given antigen, causing neutralization or opsonization of pathogens. Antibodies comprise the secreted form of a B cells surface antigen receptor (BCR) and bind to exactly the same antigen. Once a B cell encounters its matching antigen and receives a signal from Th cells, it becomes activated and differentiates into an antibody-producing plasma or memory B cell. Clonal expansion, the proliferation of antigen-specific B cells in response to antigen-stimulation, increases the number of rare antigen-specific cells. Upon activation, B cells are able to change the Ig class of their antibodies, thereby producing antibodies with different biological effector functions. In addition to antigens that can activate B cells only in combination with T cell signals, T independent antigens trigger B cells to produce antibodies without the presence of T cells.

In contrast to other immune cells, mature B cell function is not strictly zinc-restricted. However, lymphopoiesis and pre-B cell development are mainly affected by zinc deprivation due to higher levels of apoptosis, thereby leading to a depletion of the naive B cell fraction [21]. This phenomenon also occurs in elderly, marginally zinc deficient human subjects where B cell numbers are observed to decline with age [176]. Mature B cells, on the other hand, are more resistant to apoptosis, possibly due to the higher level of the anti-apoptotic molecule Bcl-2 [21].

Indicators for the correlation between zinc level and antibody production are somewhat contradictory. Zinc deprivation in mice was demonstrated to reduce the amount of specific antibody-producing B cells after immunization with a T cell-dependent antigen (sheep red blood cells), whereas the amount of antibodies produced per activated B cells remained stable [177]. This leads to the conclusion that residual splenic B cells remain reasonably functional during zinc deficiency. Nonetheless, the antibody-mediated response is impaired, basically due to reduced B cell numbers.

Accordingly, elderly subjects respond more poorly to vaccination with several antigens [178]. Also, hemodialysed patients who show a high incidence of zinc-deficiency, reveal reduced responses to vaccination [179]. In a vaccination study against diphtheria in hemodialysed patients, the response to the applied vaccine was related to serum zinc deficiency. Whereas nonresponders to the vaccine had significantly reduced serum zinc levels, responders showed similar zinc levels as age-matched controls [180]. Other studies that focused on the insufficient immune response to vaccination of hemodialysed patients reported impaired vaccination response rates towards hepatitis B, influenza, tetanus, diphtheria and others [181]. Based on these findings the idea of supplementing individuals with zinc in order to enhance responses to vaccination is obvious. However, numerous vaccination studies with additional zinc supplements did not reveal an increased antibody titer against the vaccine [182-184]. Exemplary, oral supplementation with zinc (400 mg/day) or zinc plus arginine (4 g/day) for 60 days starting 15 days before influenza vaccination was ineffective in inducing or ameliorating the antibody response after influenza vaccination in elderly

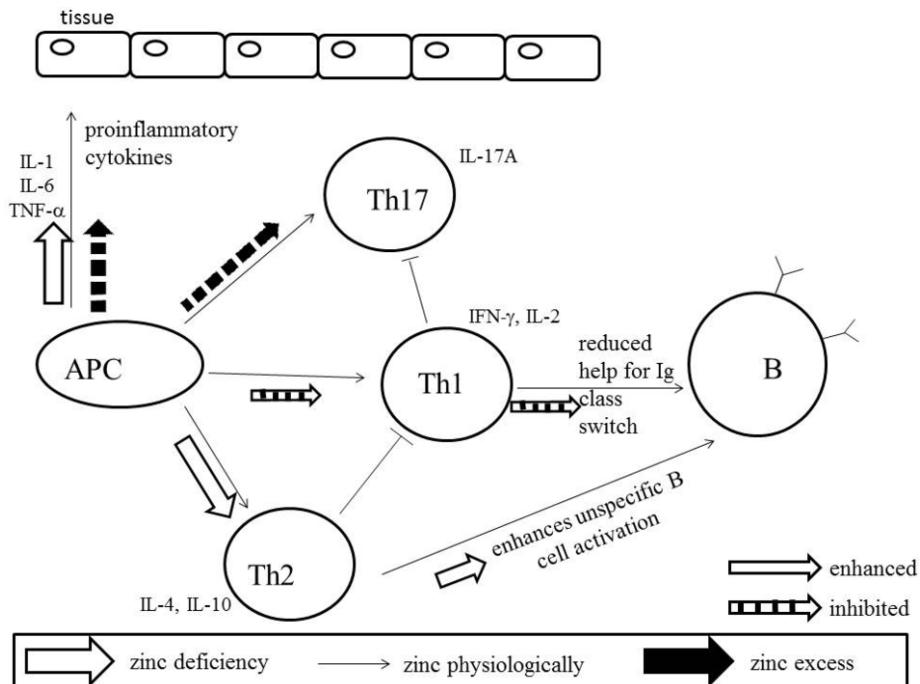


Figure 2. Influence of zinc status on immune function. Zinc deficiency increases the expression of proinflammatory cytokines and modulates the Th1/Th2 balance by reducing the Th1 type cytokines IFN- γ and IL-2. This reduces T cell help for immunoglobulin class switch and causes unspecific B cell activation by enhanced production of Th2 cytokines. Excessive zinc suppresses the expression of proinflammatory cytokines and inhibits the development of Th17 cells. Solid lines indicate pathways leading to generation of selected cytokines. Dotted lines represent pathways which lead to inhibition of cytokine generation. APC represents antigen-presenting cell types and secreted cytokines, Th1 represent activated Th1 type T cells and secreted cytokines. Th2 represents activated Th2 type T-cells and secreted cytokines, Th17 represents T17 type T cells and secreted cytokines, B-cell represents B-cell lineages. Adapted from Haase and Rink [185].

subjects [186]. However, in the majority of these studies zinc may have been applied in excessively high concentrations, thereby suppressing Th immunity (discussed above) which is required for B cell-induced antibody production. Furthermore, supplementation of elderly subjects with higher zinc dosages (30 mg/d) resulted in diminished B cell counts compared to both the low zinc dosage treated (15 mg/d) and the placebo treated control group [187]. Another reason for the missing improving zinc-effect on vaccination might be the fact that the duration of zinc administration prior to vaccination was insufficient. As B cell activation depends mostly on Th cell stimuli, restoring adequate zinc levels for proper T helper cell function needs to be achieved. Yet, this may require a longer period of zinc supplementation than provided in these cited studies. Correspondingly, Duchateau and colleagues started zinc administration one month prior to vaccination and the IgG response to vaccination did indeed improve [188].

Interestingly, a vaccination study using inactivated cholera toxin in young children showed increased levels of vibriocidal antibodies when individuals were supplemented with zinc every day for 42 days starting 3 weeks prior vaccination. But zinc supplementation did not affect the production of vaccine-specific IgG and IgA antibodies. The effect of zinc supplementation on the vibridical responses was

particularly evident in the group of zinc deficient children, whereas the responses among zinc sufficient children with zinc supplementation resembled the control group without zinc supplementation [136]. Hence, zinc supplementation may benefit the response to vaccination in zinc deficient individuals. Nevertheless, the duration and doses of zinc supplementation should be considered carefully because excessive zinc levels might inhibit T cell function or induce copper deficiency anaemia, and, consequently, result in reduced B cell antibody production.

Dropping B cell counts in elderly are accompanied by increasing IgA and IgG antibody subclasses [176]. This may possibly indicate an enhanced production of nonspecific antibodies in these individuals, linking the impaired vaccination response to a loss of antibody affinity in elderly individuals. In addition, an increase in organ-specific and non-organ specific autoantibodies was found in elderly individuals. But, whereas the latter increases with age, interestingly, subjects older than 90 years show lower levels of organ-specific autoantibodies than do younger elderly [178].

Hence, whereas the number of B cells and specific antibodies (e.g., in response to vaccination) decreases with age, the total and nonspecific IgGs and autoantibodies increase, causing hampered B cell function. Moreover, some B cells develop enhanced age-dependent clonal expansion, which may be connected to a higher incidence of lymphocyte malignancies with advancing age [178]. However, although the mentioned phenomenon in elderly is attributed to zinc deficiency, it has to be considered that other factors apart from zinc deficiency occur during aging, which may also contribute to disturbed B cell function.

Figure 2 summarizes the influence of zinc status on immune function, comparing the effects of zinc deficiency, zinc excess, and physiological zinc levels.

Recently, an adjuvant effect of zinc oxide on the immune response in mice was demonstrated [189]. Mice were intraperitoneally administered with ovalbumin (OVA) either with or without varying doses of zinc oxide. Simultaneous administration of OVA and zinc induced a stronger increase in anti-OVA IgG production accompanied by enhanced antigen-specific splenocyte proliferation compared to that of OVA alone. Enhanced IgG production was associated with increased Th2 responses, indicated by elevated levels of Th2 cytokines IL-4 and IL-5, which in turn promote B cell differentiation, Ig production and isotype switch. The enhancing effect of zinc on Th2 response was comparable to the effect of aluminium hydroxide, a widely used adjuvant. Still, the advantage of zinc as an adjuvant needs to be further investigated, given that zinc application is associated with increased Th1 response in most cases.

However, it will be an interesting task to further investigate the effect of zinc supplementation, either on a pharmaceutical level or as an adjuvant, in order to clarify the impact of zinc supplementation on humoral immunity. The following section deals with the effect on zinc on immune regulation.

5. Impact of Zinc in Immune Regulation

5.1. Cytokines

Cytokines act as immunomodulating agents within the immune system. Zinc has the ability to interfere in this complex signaling network by modulating the mRNA expression of different cytokines [96; 97]. In addition, zinc homeostasis influences the signal transduction of cytokines in their target cells [166]. As mentioned before,

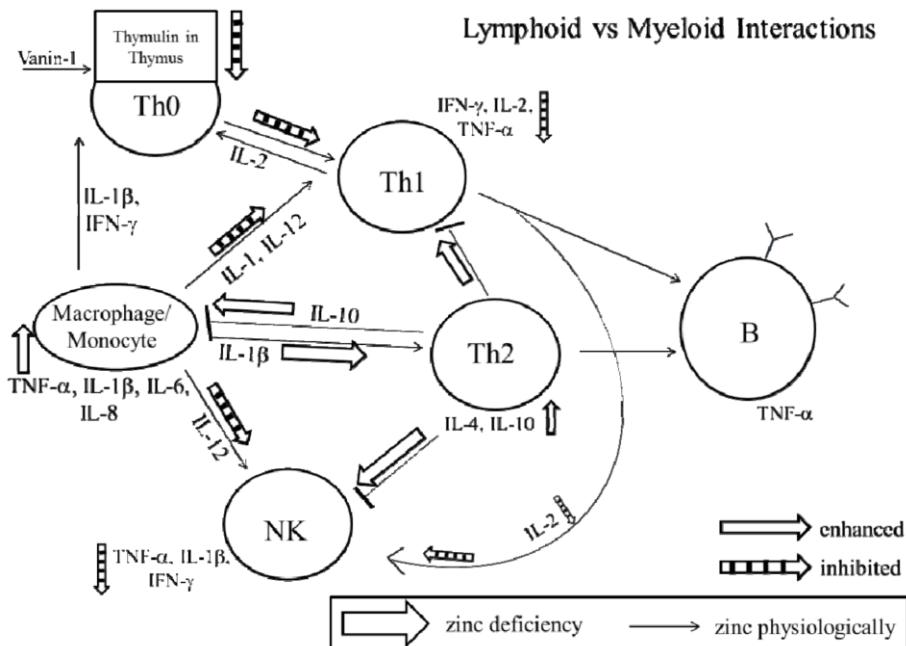


Figure 3. Landscape of zinc effect on immune cells. Zinc is an integral part of a thymic hormone molecule, thymulin. Thymulin is required for maturation of T-cells. Zinc deficiency-induced decrease in thymulin activity is associated with decreased maturation of T-cells and Th1 production of IL-2 and IFN- γ . Decreased IL-2 leads to decreased NK- and T cytolytic cell activities. Macrophages/monocytes produce IL-12 (a zinc dependent cytokine) which, along with IFN- γ promotes killing of parasites, viruses and bacteria. Th2 cytokines, in general are not affected by zinc deficiency except IL-10 which may be increased in zinc deficient elderly subjects. Increased IL-10 from Th2 cells further affects Th1 functions adversely. Thus, in zinc deficiency there is a shift from Th1 to Th2 functions and cell-mediated immune functions are impaired. Zinc deficiency also leads to stress and activation of macrophages/monocytes, resulting in increased generation of inflammatory cytokines, IL-1 β , IL-6, IL-8 and TNF- α . Solid lines indicate pathways leading to generation of selected cytokines and dotted lines represent pathways which lead to inhibition of cytokine generation. NK represent natural-killer cells; Th1 represent activated Th1 type T cells and secreted cytokines (small triangles); Th2 represents activated Th2 type T cells and secreted cytokines; B-cell represents B-cell lineages and associated immunoglobulins. Adapted from Shankar and Prasad [72].

cytokine production is affected by altered zinc homeostasis. Since cytokines are key players in intercellular communication, zinc is involved in regulating the interplay between innate and adaptive immunity.

In detail, zinc influences the expression of pro-inflammatory cytokines. Zinc supplementation in PBMC enhances the mRNA production and release of the monokines IL-1, IL-6 and TNF- α , and synchronous application of zinc and LPS results in the production of a large amount of monokines [152; 190; 191]. In contrast, some reports suggest that zinc treatment suppresses the formation of pro-inflammatory cytokines [97; 101; 192]. A possible explanation for these diverging effects might be a concentration-dependent effect of zinc on different signaling molecules involved in monokine expression. Low zinc concentrations mimic and trigger the zinc signal, which is usually involved in monokine production of monocytes in response to LPS [86]. On the contrary, high zinc levels inhibit the cyclic nucleotide phosphodiesterase (PDE) and suppress subsequent activation of protein kinase A, which results in abrogated cytokine production [99; 100]. The zinc-related suppression of inflammatory

cytokines is additionally associated with an induction of A20-mediated inhibition of NF κ B. A20 expression is NF κ B dependent and has been shown to negatively influence the LPS signaling, thereby providing an autoregulatory feedback loop that protects cells from LPS- and TNF- α - induced cytotoxicity [193]. Zinc was shown to induce A20, which results in suppressed TNF- α expression [110].

Cytokine production in PBMCs after zinc supplementation does not only affect monokine levels, but also T cell cytokine levels (e.g. IFN- γ) are elevated by zinc [95]. Since this effect depends on the presence of monocytes and is based on direct cell to cell contact and zinc-mediated production of the monokines IL-1 and IL-6, the cytokine production of T cells measured in PBMC is rather due to an indirect effect based on the elevated T cell activating monokine levels.

Still, zinc effects on T cell cytokine expression turn out to likewise depend on zinc concentration. Zinc deprivation abrogates the expression of Th1 cytokines such as IFN- γ and IL-2, while maintaining the expression of Th2 cytokines [116; 133]. Marginally enhanced zinc levels, on the other hand, ameliorate the production of IFN- γ in T cells [147] and were recently shown to promote the expression of IL-2 and IFN- γ mRNA via zinc-mediated activation of the transcription factor CREB [194]. In contrast, high zinc concentrations suppress the production of IFN- γ , as demonstrated in a human T cell line upon activation and in the abrogated IL1- β -stimulated IFN- γ secretion in primary T cells [95].

Since IL-2 and IFN- γ play key roles in the immune response by mediating the expansion and maintenance of thymocytes and peripheral T cell populations as well as the generation of immune tolerance and the activation of other immune cells, reduced zinc levels affect the complex communication network and interaction between hematopoiesis, innate and adaptive immunity via modulation of cytokine levels and signaling. Figure 3 presents an overview about the immune regulatory effect of zinc on the cytokine production of different immune cells.

5.2. Other Immune Regulatory Elements

Nitric oxide (NO) plays an important role in the cellular and vascular response to inflammation and is hence considered to be an immunologically important effector biomolecule, particularly in the innate immunity [195]. NO production is regulated through the inducible NO synthase (iNOS) in response to infections, injurious agents, and proinflammatory cytokines produced by the host [195-197]. NO, derived from iNOS or other NO donors, appears to be linked to intracellular zinc homeostasis, since NO dependent S-nitrosylation of cysteins in MT causes zinc release [198]. The proinflammatory cytokine-induced upregulation of iNOS in hepatocytes results in the generation of a large amount of NO, concomitant with increased MT expression [124].

Subsequently, NO promotes zinc release from MT. Free zinc, in turn, is considered to repress iNOS, thereby counteracting NO production [199]. In accordance, zinc deficient rodents showed increased iNOS expression [200], perhaps because MT-bound zinc was not available. Increased NO levels may therefore contribute to inflammatory processes during zinc deficiency. Furthermore, the LPS-induced proinflammatory cytokine IL-1 β , an activator of iNOS and NO production, led to increased ZIP14 mRNA transcription and enhanced zinc transport in hepatocytes in wild-type, but not in iNOS $^{-/-}$ cells [201]. Thus, NO is involved in hepatocytic zinc accumulation, promoting hypozincemia during inflammation and sepsis.

Due to their bacteriocidal activity, ROS production via oxidative burst is indispensable for innate immunity. However, overproduction of ROS results in oxidative stress, which can also be induced or further aggravated by ROS formation as an unavoidable byproduct of cellular respiration and due to interaction of ionizing radiation with biological molecules as seen during peroxisomal fatty acid metabolism [202; 203]. Based on their strongly oxidative nature, ROS are involved in the damage of molecules and cell structures. ROS are therefore considered as cellular stressors that contribute to a variety of pathological processes such as various cancer, neurodegenerative, viral, toxic or inflammatory diseases leading to endothelial and epithelial damage [204]. Zinc in physiological concentrations elicits antioxidant effects by protecting biological structures and affecting gene expression of proteins involved in the stress response [205]. In contrast, both exceedingly high zinc levels and zinc deficiency promote oxidative stress by eliciting pro-oxidative functions [206]. The role of zinc as an antioxidant and anti-inflammatory agent is summarized in figure 4. More about the biochemistry of zinc and its molecular function in controlling redox metabolism is depicted in chapter 4.

Apart from this, zinc affects the protection against viral infection. Recently, zinc was demonstrated to directly inhibit the *in vitro* activity of the coronavirus and arterivirus RNA polymerase and thus blocks the replication of these viruses [207]. Increased intracellular zinc levels were consistently associated with impaired replication of other viruses, e.g. influenza [208]. Furthermore, zinc supplementation was associated with enhanced type I IFN receptors 2 (IFNAR2) expression in U937 cells, resulting in ameliorated IFN- α -induced antiviral capacity [209]. Consistently with this finding, zinc was shown to tenfold potentiate the antiviral action of IFN- α in cells challenged with rhinovirus [210]. In elderly individuals decreased secretion of IFN- α was observed after virus stimulation *in vitro* and zinc supplementation fully reconstituted the diminished IFN- α secretion [211].

Recently published results of a placebo-controlled trial of zinc lozenges for the treatment of common cold revealed a shorter mean overall duration of cold in the zinc group compared to placebo group (4.0 vs 7.1 d, p<.0001) and shorter duration of cough (2.1 vs 5.0 d, p<.0001) and nasal discharge (3.0 vs 4.5 d, p=.02) [212]. Symptoms severity scores were decreased significantly. Furthermore, plasma IL-1ra and sICAM-1 levels decreased significantly in the zinc group. IL-1ra is an anti-inflammatory cytokine which functions as a specific inhibitor of IL-1 α and IL-1 β inflammatory cytokines. Decreased IL-1ra levels in the zinc group indicate that overall inflammation was diminished in this group. Plasma sICAM-1 was also decreased in zinc treated subjects. Human rhinovirus type 14 “docks” with ICAM-1 on the surface of somatic cells. Thus, zinc may act as an antiviral agent by reducing ICAM-1 levels. Another possibility is that zinc ions may form a complex with ICAM-1, preventing the binding of rhinovirus to cells.

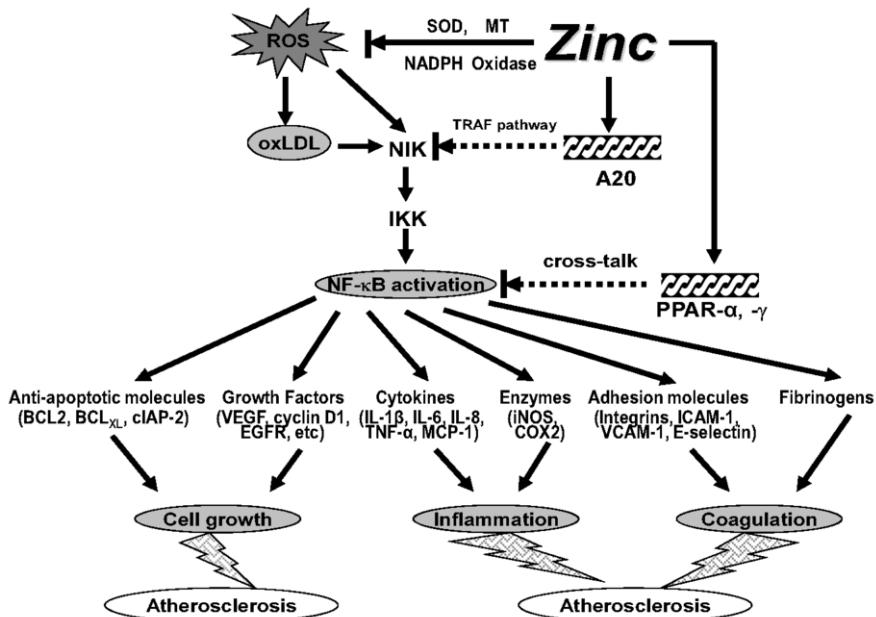


Figure 4. Zinc as an antioxidant and anti-inflammatory agent. Zinc decreases ROS (reactive oxygen species) by several mechanisms. Zinc is an inhibitor of NADPH oxidase, a requirement for superoxide dismutase (SOD), and it induces MT (metallothionein) which is very effective in decreasing OH. ROS activates NF- κ B which, in turn, activates growth factors and anti-apoptotic molecules resulting in cancer cell proliferation. NF- κ B activation also induces the generation of inflammatory cytokines and adhesion molecules. One mechanism by which zinc reduces inflammatory cytokine production involves the zinc-induced up-regulation of a zinc-finger protein, A20, which inhibits NF- κ B activation via TRAF pathway. Zinc, thus not only functions as an antioxidant but is also an anti-inflammatory agent. Arrows represent directional flow for events presented in this cartoon. Solid arrows represent events leading to selected events and dotted lines represent inhibition of selected events. Adapted from Bao et al [102].

Although zinc appears to be a potent suppressor of viral infection, further investigation is needed to elucidate the capacity of zinc to protect the body against virus-related diseases.

6. Conclusion and Future Perspectives

Zinc ions are indispensable for immune function. The development and functional effectiveness of virtually all immune cells is modulated by zinc. These effects can either be direct, for example by affecting gene expression of effector molecules, or indirect, by influencing responsiveness of effector cells or by regulating cell communication. Due to its multiple functions in immune cell activity, disturbances of intracellular zinc levels deeply affect the immune cell activity. Therefore, the cellular zinc homeostasis has to be tightly controlled. During zinc deprivation, the immune response is impaired based on an insufficient pathogen defense combined with dysregulated immune cell activity. This interaction provokes the development of many diseases. Physiological zinc concentrations are required for adequate immunity, which, during zinc deficiencies, may be supplemented in order to restore the physiological level. This phenomenon is partly related to the anti-inflammatory and anti-oxidative

character of zinc ions. However, excessive zinc levels are associated with immune suppression owing to the inhibition of leukocyte function. As a consequence, zinc supplementation might help to restore the immune function of patients with low serum zinc concentrations, but must be well adjusted to the actual requirement. If zinc concentration much exceeds the physiological level, however, zinc may also influence immune function adversely.

Substantial research progress has been achieved over the last years on the mechanisms by which zinc acts at the molecular level. Especially its role as a signaling molecule is well established. Yet, many questions remain open regarding the opposing effects of varying zinc concentrations on the distinct signaling pathways in different cell types. In addition, further research is needed in the future to elucidate the epigenetically impact of zinc and its effect on immune cells, the more as the overall influence of zinc homeostasis on immune cell reactivity and the resulting immune response.

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11. Zinc and Infectious Diseases

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Abstract. Zinc is an essential micronutrient critical to the maintenance of a healthy immune system. Zinc deficiency is often linked with an increased risk for infectious diseases especially among at-risk populations such as young children and pregnant women. Supplementation with zinc has been shown to prevent and treat diarrhea among children under 5 years of age decreasing both diarrhea morbidity and mortality. Zinc deficiency is also correlated with risk for respiratory infections, but the benefit of supplementation appears to be limited to more severe episodes and populations with high rates of zinc deficiency. While there is evidence suggesting a correlation between zinc deficiency and the prevalence of malaria, measles, HIV, and tuberculosis, few studies have shown a benefit of supplementation for either prevention or treatment of these infections. The World Health Organization currently recommends zinc supplementation for the treatment of diarrhea among children under 5 years of age.

Keywords. zinc, infectious disease, diarrhea, pneumonia, treatment

Introduction

Zinc is an essential micronutrient required for cell growth, differentiation and the maintenance of a healthy immune system [1, 2]. Human zinc deficiency was first recognized by Prasad et al. who observed a cohort of adolescent boys in Egypt with extremely poor growth, delayed maturation, and an increased risk for infectious diseases [3]. Since these initial reports, a number of observational studies have linked individual zinc status to one's susceptibility to increased rates of infection [4-6]. Further research for several infectious diseases, such as diarrhea, pneumonia, and the common cold, have explored the effects of supplementation on prevention and treatment of disease.

Infectious diseases remain a leading cause of morbidity and mortality especially among young children [7, 8]. Globally, the countries with the highest proportion of Disability Adjusted Life Years (DALYs) attributable to infection also have the highest rates of undernutrition and are at the greatest risk of zinc deficiency [7, 8]. Understanding the link between micronutrient status, in this case zinc deficiency, and susceptibility to infection is critical on a population level to identify joint risk factors

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and on an individual level to recognize the opportunity for improved zinc nutriture whilst also preventing or treating several infectious diseases.

In this chapter we present the evidence linking deficiency with selected infectious diseases and the role of zinc supplementation for the prevention and treatment of these illnesses. A full review of zinc and immune function has already been provided in Chapter 10. Here we highlight the most widely studied infectious diseases found to be potentially influenced by individual zinc status.

1. Diarrhea

Diarrhea causes rapid transit in the gut, breakdown of absorptive mucosa and damage to specific transporters, resulting in poor absorption of nutrients, including zinc [9]. Studies conducted during the late 1970s and 1980s linked diarrheal illness to the loss of endogenous zinc [10-12]. Moreover, children with low plasma zinc, an indicator of zinc deficiency, are more susceptible to diarrhea pathogens, propagating a cycle of deficiency and infection [13].

1.1. Prevention of Diarrhea

There is extensive evidence supporting the efficacy of zinc supplementation for the prevention of childhood diarrhea. Early trials of preventive zinc supplementation were conducted in response to studies documenting that diarrhea is part of the clinical manifestations of severe zinc deficiency disorders and in zinc-deficient animal experiments [14-16]; preventive supplementation trials were also spurred by studies finding associations between diarrhea and indicators of human zinc deficiency [12, 17]. Preventive zinc supplementation has been studied via two delivery mechanisms—daily or weekly routine supplementation over a prolonged period and short course supplementation. Routine supplementation entails the provision of zinc supplements for a period of 3-15 months and is not initiated in relation to an illness [18]. Short course supplementation involves the provision of zinc supplements typically for 10-14 days for the treatment of diarrhea with follow-up extending for several months post supplementation [18].

1.1.1 Prevention of Diarrhea with Routine Supplementation

Initial randomized controlled trials (RCTs) linked routine supplementation with elemental zinc to reduced incidence and prevalence of diarrhea among children in developing countries [18]. A community-based trial in India reported an 8% (95% CI: -2-18%) decrease in incident diarrhea and a 6% (95% CI: -10-20%) decrease in prevalent diarrhea among children 6-41 mo of age randomized to receive 10 mg of zinc per day [19]. In the subgroup of children with lower plasma zinc levels (<9.18 µmol/L), diarrhea incidence was decreased by 17% (95% CI: 1-30%) among zinc-supplemented children. These trends were reinforced by additional community-based studies conducted in Vietnam [20], Mexico [21], and Guatemala [22].

In 1999, the Zinc Investigators' Collaborative Group published the first meta-analysis of RCTs of routine zinc supplementation [23]. This pooled analysis, which

included routine supplementation in 7 studies providing 1-2 times the recommended daily allowance (RDA) of elemental zinc 5-7 times per week, found an 18% (95% CI: 7-28%) reduction in diarrhea incidence, a 25% (95% CI: 12-37%) decrease in diarrhea prevalence, and a 33% (95% CI: -6-58%) reduction in persistent diarrhea episodes among supplemented children compared to children who received placebo.

Subsequent RCTs were conducted to reaffirm the observed efficacy of routine zinc supplementation in various populations. An updated meta-analysis of 15 RCTs was also published in 2007 [24]. Included studies provided 15-140 mg of zinc per week for ≥ 3 months. The results of the pooled analysis indicated a 14% (95% CI: 7-21%) reduction in incident diarrhea among children receiving zinc supplements compared to placebo. A sub analysis of three studies yielded a pooled reduction in persistent diarrhea of 25% (95% CI: 2-43%) among routinely supplemented children.

Although findings supporting the efficacy of routine zinc supplementation for the prevention of diarrhea have been consistent overall, studies have illustrated modification of the effect by age. Sazawal et al. reported a 27% decrease in diarrhea incidence among routinely supplemented children ≥ 12 mo of age and no reduction among similarly supplemented children 6-11 mo of age [19]. Pooled subgroup analyses conducted by the Zinc Investigators' Collaborative Group found that effect sizes for the reduction in incident diarrhea among routinely zinc-supplemented children were consistent when comparing those < 12 mo with age with those ≥ 12 mo of age [23].

1.1.2. Prevention of Diarrhea with Short Course Supplementation

An early RCT of zinc supplementation in Bangladesh observed a 38% reduction in diarrhea incidence during the 3-month period following 2 weeks of zinc for the treatment of persistent diarrhea [18]. This finding suggested the potential benefit of short-course zinc supplementation administered during and shortly following a diarrheal episode.

The 1999 meta-analysis published by the Zinc Investigators' Collaborative Group included three RCTs providing short course zinc supplementation with 2-4 times the daily RDA for two weeks following the onset of an episode of acute or persistent diarrhea [23]. The pooled analysis showed an 11% (95% CI: -28-38%) decrease in diarrhea incidence and a 34% (95% CI: 17-48%) decrease in diarrhea prevalence. In 2002, Bhandari et al. published the results of an RCT in which children 6-30 mo of age living in the urban slums of New Delhi were randomized to receive 20 mg of elemental zinc daily (10 mg to infants) or placebo [25]. They reported a reduction in both diarrheal incidence (12%; 95% CI: 5-18%) and mean prevalence (difference in means: -1.30; 95% CI: -0.61 to -2.0). Additionally, the reported risk of recurrent diarrhea, defined as >6 diarrheal episodes during the observation period, was 49% (95% CI: 23-64%) lower among zinc-supplemented children compared to those receiving placebo. The risk of hospitalization due to diarrhea was also reduced by 26% (95% CI: -27-54%) in the zinc supplementation group.

Findings supporting the efficacy of preventive short course zinc supplementation are inconsistent across age groups. Bhandari et al. reported that reductions in incident diarrhea were only statistically significant in children ≥ 12 mo of age [25]. Similarly, in a trial conducted among infants < 6 mo of age in India, Ethiopia, and Pakistan, short course supplementation administered at diarrhea onset did not show a benefit on subsequent morbidity in the 8 weeks following the index diarrhea episode and 14 days of supplementation [26].

1.2. Treatment of Diarrhea

During the late 1980s and 1990s, RCTs of zinc supplementation were conducted in response to mounting evidence providing a physiologic basis for the observed link between zinc deficiency and infectious diarrhea during childhood [18, 23, 27]. These trials demonstrated the efficacy of zinc as a treatment for diarrhea, and in 2004, the World Health Organization issued a global recommendation for daily supplementation with 20 mg of zinc in children \geq 6 mo and 10 mg of zinc in infants under 6 mo for 10–14 days upon diarrhea onset [28] (Table 1).

Sachdev et al. conducted the earliest study evaluating the efficacy of zinc as a potential treatment for diarrhea infection [18, 29]. The study, which was conducted in India on children 6–18 mo of age hospitalized for acute diarrhea, randomized 50 children to receive placebo or 40 mg of elemental zinc per day. Among zinc-treated children, they observed a 9% decrease in the duration of diarrhea and an 18% decrease in stool frequency. The effect was modified by level of rectal mucosal zinc, such that children with lower levels of tissue-zinc experienced greater reductions in diarrhea duration and stool frequency. None of these findings were statistically significant.

Additional studies assessing the efficacy of zinc treatment among young children with acute diarrhea included a hospital-based RCT in Bangladesh [30], and community-based RCTs conducted in India [31], Bangladesh [32], and Indonesia [33]. Of these early studies, all observed a reduction in the duration of acute diarrheal episodes among zinc-treated children, as well as decreased severity as indicated by lower stool frequency. Studies also found that episodes lasting longer than 7 days occurred 25–43% less often when children were treated with zinc compared to those who received placebo [18]. In addition, one hospital-based study observed a decrease in stool output among zinc-treated patients [30].

Early studies also observed that nutritional status modified the effect of zinc treatment on the duration of acute diarrhea. In a community-based trial of 937 children in India, stunted children experienced greater reductions in the duration and severity of diarrheal episodes [31]. The same trend was observed in the subgroup of children with low plasma zinc concentration ($<14 \mu\text{mol/L}$) in a hospital-based trial in Bangladesh [30]. In an RCT in Ethiopia, Umetsu et al. also reported markedly greater reductions in diarrhea morbidity and frequency among stunted zinc-supplemented children compared to non-stunted zinc-supplemented children [34].

In addition to initial data on the efficacy of therapeutic zinc for acute diarrhea outcomes, evidence supporting its efficacy in the treatment of persistent diarrhea was also reported. Sachdev et al. were the first to report on this subject in an RCT of 40 children 6–18 mo of age hospitalized for persistent diarrhea [18, 35]. The duration of diarrhea was reduced by 19% among children randomized to receive 40 mg of elemental zinc per day (not statistically significant). A second hospital-based study of children with persistent diarrhea conducted in Bangladesh also suggested a decrease in the duration of diarrhea among zinc-treated children [30]. This and an additional hospital-based study, conducted in Pakistan [36], suggested stronger efficacy of zinc treatment among children with poorer nutrition status and lower plasma zinc levels. These trends were also observed by a community-based RCT of 275 children 6–36 mo of age in Peru [37].

In 2000, the Zinc Investigators' Collaborative Group published the first meta-analysis summarizing the findings of studies assessing the therapeutic effects of zinc on acute and persistent diarrhea in children under five in developing countries [38]. The

pooled analysis of published and unpublished RCTs included three and four studies on acute and persistent diarrhea, respectively. Among the key findings, the probability of continuing diarrhea was 15% (95% CI: 8-22%) lower for acute episodes and 24% (95% CI: 8-38%) lower for persistent episodes among zinc-treated children. Mean duration was shorter for both acute (16%; 95% CI: 7-26%) and persistent (29%; 95% CI: 6-53%) episodes, and the overall rate of prolonged episodes was 27% (95% CI: 2-45%) lower among children receiving zinc.

Subsequent RCTs aiming to determine the consistency and generalizability of earlier findings in a variety of populations were published between 2000 and 2010 [39-46]. A number of trials with cluster-randomized designs were also conducted in Bangladesh [47] and India [48]. The results of cluster-randomized trials confirmed those of randomized placebo-controlled trials. Baqui et al. observed a 24% (95% CI: 10-35%) shorter duration and a 15% (95% CI: 4-24%) decreased incidence of diarrhea among children from zinc intervention clusters in a study of over 8000 children in rural Bangladesh [47]. In a comparative trial of 20,000 caregivers in India, Bhandari et al. found that in communities with access to zinc for the treatment of diarrhea children had reduced diarrhea prevalence rates for both 24-hour (25% reduction, 95% CI: 9-38%) and 2-week prevalence (44% reduction, 95% CI: 25-59%). Hospitalizations were also reduced by 59% (95% CI: 43-71%) [48].

A Cochrane review of the impact of therapeutic zinc on diarrheal morbidity was published in 2008 and supported previously observed trends in episode duration and stool frequency [49]. An updated meta-analysis published in 2009 further substantiated the evidence supporting the efficacy of zinc for the treatment of childhood diarrhea [50]. In pooled analyses of 14 RCTs assessing acute episodes and 5 RCTs assessing persistent episodes, therapeutic zinc decreased the duration of diarrhea in children under five by 0.5 and 0.68 days, respectively ($p<0.05$). An added analysis of six studies showed a 32% (95% CI: -1-54%) reduction in the proportion of acute episodes persisting beyond 7 days among zinc-treated children. A pooled estimate derived from four studies evaluating recovery from persistent diarrhea illustrated a 21% (95% CI: 4-35%) decrease in the probability of continuing diarrhea on any given day among zinc-treated compared to control children.

While findings supporting the efficacy of therapeutic zinc have been largely consistent across studies, data have been published indicating effect modification by age. The first RCT specifically assessing the efficacy of zinc treatment among infants 1-5 mo of age did not observe any differences in the duration or severity of acute episodes comparing controls to individuals receiving zinc daily [51]. A subsequent RCT conducted in Pakistan, India, and Ethiopia also reported no effect on diarrhea duration among infants < 6 mo of age [52]. In contrast, two cluster-randomized trials in Bangladesh and India observed trends for shorter duration and reduced incidence among zinc-treated infants <6 mo of age [53, 54]; however, the reported effect sizes were not statistically significant in Bangladesh as a result of small sample sizes specifically within this age group.

1.3. Diarrhea hospitalizations and mortality

Therapeutic zinc has been reported to reduce hospital admissions for diarrhea in two cluster-randomized trials [47, 48]. A meta-analysis of these trials yielded a 23% (95% CI: 15-31%) reduction in diarrhea hospitalizations among communities receiving zinc intervention compared to control clusters [55].

The impact of therapeutic zinc on mortality was first reported by Baqui et al. in 2002 [47]. In a cluster-randomized controlled trial of over 8000 children in rural Bangladesh, the rate of non-injury related deaths was 51% (95% CI: 6-75%) lower in communities randomized to zinc treatment in addition to standard Oral Rehydration Salts (ORS). Although additional studies also counted deaths, none have been adequately powered to report an effect size on mortality [55].

Studies have also suggested an association between preventive zinc supplementation and reductions in all-cause mortality. A randomized controlled trial conducted in Pemba, Zanzibar reported a non-significant 7% decrease in all-cause mortality among zinc-supplemented children compared to placebo [56]. A community-based cluster-randomized controlled trial in Nepal also reported a trend toward a reduction in mortality among children ≥ 12 mo receiving zinc compared to placebo [57]. Neither trial observed a reduction in mortality among children under 12 mo of age.

1.4 Etiologic Specific Effects

In-vitro studies and RCTs have documented the effects of both preventive and therapeutic zinc by diarrhea etiology. Overall, though the etiologic-specific evidence is not vast, the findings are largely consistent across etiologic agents.

A pooled analysis of three RCTs of preventive zinc supplementation showed a 13% (95% CI: -19-36%) decrease in dysenteric illness among children receiving zinc supplements [23]. This result was supported by a second meta-analysis of five routine zinc supplementation trials, which showed a 15% (95% CI: 5-25%) reduction in severe dysenteric diarrhea [24]. An RCT of therapeutic zinc among *Shigella flexneri*-infected children hospitalized in Bangladesh showed that 14 days of zinc supplementation in combination with antimicrobial treatment boosted immune response [58]. A subsequent study of short course supplementation in shigella-infected children in Bangladesh reported faster recovery and a reduction in episode recurrence among supplemented individuals [42].

In a recent study, decreased episode duration and stool output were also reported among cholera-infected children randomized to receive 30 mg of zinc per day in a hospital-based trial in Bangladesh [59].

Table 1. Summary of effect sizes reported by key meta-analyses on the effect of preventive and therapeutic zinc on diarrhea morbidity and mortality outcomes.

Outcome Measure	Effect Size (95% CI)	Number of Studies Year of Publication [Ref]
Routine Zinc Supplementation		
Diarrhea Incidence	18% reduction (7-28%)	7 1999 [23]
	14% reduction (7-21%)	15 2007 [24]
Diarrhea Prevalence	25% reduction (12-37%)	7 1999 [23]
Persistent Diarrhea	33% reduction (-6-58%)	5 1999 [23]
	25% reduction (2-43%)	3 2007 [24]
Short Course Zinc Supplementation		
Diarrhea Incidence	21% reduction (-28-38%)	3 1999 [23]
Diarrhea Prevalence	34% reduction (17-48%)	3 1999 [23]
Therapeutic Zinc		
Mean Duration of Acute Episodes	16% reduction (7-26%)	3 2000 [38]
	reduction by 0.5 d (0.18-0.82)	14 2009 [50]
Mean Duration of Persistent Episodes	27% reduction (2-45%)	4 2000 [38]
	reduction by 0.68 d (-0.01-0.64)	5 2009 [50]
Diarrhea Hospitalizations	23% reduction (15-31%)	2 2010 [55]

2. Respiratory Infections

Respiratory tract infections account for an enormous burden of morbidity and mortality across all age groups around the world. The most frequent infection of the upper airway is the common cold, an acute self-limited viral infection of the nasopharynx that causes discomfort and malaise, missed school days, reduced economic productivity, and can evolve into more severe bacterial infections of the respiratory tract [60]. Acute lower respiratory infections (ALRI), including pneumonia and bronchiolitis, are less common than extra-thoracic infections but are much more likely to cause severe morbidity and mortality. Among children under the age of five years, ALRI is the single most important cause of death globally [61].

There is an extensive literature documenting the biological, clinical, and epidemiological associations between zinc deficiency and human susceptibility to infections of the respiratory tract. Zinc exerts a range of anti-oxidant (see chapter 4) and cytoprotective (see chapter 5) properties that maintain the integrity of the respiratory epithelium for protection against infectious insults [62]. ALRI severity may be a function of the exuberance of the host inflammatory response [63], suggesting that zinc could attenuate respiratory disease pathogenesis by modulating pro-inflammatory cytokine production [64]. Zinc is also postulated to have pathogen-specific effects; for example, zinc has been shown to inhibit the replication of rhinovirus [65], the major cause of the common cold, and respiratory syncytial virus (RSV) [66], an important cause of ALRI in children worldwide [67]. In mice, zinc depletion increased the severity of pneumococcal infection [68], a major cause of mortality among young children [69] and older adults [70]. In epidemiological studies, zinc deficiency defined by low serum zinc concentration has been associated with an increased risk of respiratory morbidity [71-73], highlighting the potential benefit of improvements in zinc nutriture in populations at risk of ALRI.

2.1 Prevention of Acute Lower Respiratory Tract Infections

The strongest evidence of the causal association between zinc status and ALRI is derived from controlled trials of oral zinc supplementation. Most published randomized controlled trials assessing the effect of zinc on the incidence of ALRI have enrolled young children in developing countries among whom there are concurrent high risks of nutritional deficiencies and infectious morbidity. Zinc deficits may influence susceptibility to ALRI in other high-risk populations (e.g., the elderly institutionalized in developed countries [73]); however, the focus of the following discussion is on childhood ALRI in community contexts because of the paucity of experimental clinical research involving other groups and other settings.

Since the late 1990s, the effect of zinc on childhood ALRI susceptibility has been studied by applying a similar methodological approach to the trials of zinc for diarrhea prevention described above; many studies have involved prospectively monitoring for both diarrhea and ALRI in the same supplemented children. An early meta-analysis of four preventive trials by the Zinc Investigators' Collaborative group in 1999 suggested that routine daily oral zinc supplementation of 1-2 times the RDA, for at least three months, significantly lowered the incidence of ALRI in young children in developing countries [23]. However, several trials subsequently conducted in South Africa [74], Peru [75], and Nepal [57] failed to confirm the benefit, such that the most recent meta-analyses have estimated a more modest overall 13-15% reduction in the incidence of ALRI, with notable heterogeneity among trials (Table 2).

Differences in baseline characteristics of enrolled children (e.g., age range, prevalence of stunting or zinc deficiency) may influence the effect of zinc; for example, Brown et al. (2009) found evidence of a greater benefit in populations with high prevalence of stunted growth, a population-level indicator of zinc deficiency [76]. An alternative explanation for divergent trial results is variation in the ALRI case definitions used as trial outcomes [77]. Estimates of the zinc effect using relatively specific ALRI case definitions (e.g., clinical evidence of lung involvement) have shown a pronounced reduction in the incidence of ALRI [77, 78], whereas there was no benefit of zinc in the prevention of ALRI when it was defined non-specifically (e.g., based on elevated respiratory rate alone) [76-78] (Table 2). Employing vague case

definitions may have obscured or diluted true effects on ALRI if many of the children included in the analysis had conditions that were not infectious or relatively non-responsive to zinc status (e.g., asthma exacerbations). Furthermore, the impact of zinc supplementation on the clinical presentation and severity of illness of childhood infections likely depends on host-pathogen interactions [79, 80]; the magnitude or direction of effect may thus vary according to specific microbial pathogens with which the child is colonized [80].

Table 2. Summary of effect sizes of key meta-analyses of randomized controlled trials of routine oral zinc supplementation for the prevention of acute lower respiratory infection (ALRI) among children under five in developing countries.

Meta-analysis	Publication Year	Number of Trials	Effect Size (95% CI)
ALRI based on variable case definitions			
Bhutta	1999	4	0.59 (0.41 – 0.83)
Aggarwal	2007	4	0.79 (0.65 – 0.95)
Brown	2009	16	0.85 (0.75 – 0.97)
Roth	2010	10	0.86 (0.74 – 1.01)
Lassi	2010	6	0.87 (0.81 – 0.94)
Physician-diagnosed ALRI based on specific case definitions (severe disease and/or clinical signs of pulmonary disease)			
Roth	2010	3	0.65 (0.52 – 0.82)
Lassi	2010	4	0.79 (0.71 – 0.88)
Physician-diagnosed ALRI based on less specific case definitions (rapid respiratory rate with or without other signs)			
Roth	2010	4	0.96 (0.86 – 1.08)
Lassi	2010	4	0.95 (0.86 – 1.06)
ALRI based on caregiver report of symptoms and signs			
Roth	2010	6	1.01 (0.91 – 1.12)
Brown	2009	7	0.99 (0.91 – 1.08)

The possible modification of the effect of zinc on ALRI susceptibility by host-, disease- or pathogen-related factors requires further investigation. Nonetheless, the accumulated evidence suggests that routine supplementation with at least one RDA oral zinc for 3 months or more among children 2 months to 5 years of age reduces the risk of moderate-severe ALRI by up to 35% in regions where the risk of zinc deficiency is high (Table 2). An important policy-relevant question is whether shorter durations of supplementation would achieve a similar benefit. There have been several large-scale cluster-randomized effectiveness trials of short-course zinc supplementation (10–14 days) to treat episodes of acute childhood diarrhea in developing countries and, as noted earlier, these trials have shown enduring beneficial reductions in diarrheal morbidity and mortality. Pooling of those studies that reported ALRI outcomes

indicated a reduction in the subsequent risk of ALRI (23% reduction, based on 3 trials) or hospitalization for ALRI (50% reduction, base on 2 trials) in the months following the treatment course. Although these results were not statistically significant and thus remain inconclusive [55], they are consistent with the evidence from long-term supplementation trials.

2.2 Treatment of Lower Respiratory Tract Infections

The primary rationale for considering a therapeutic benefit of oral zinc supplementation as an adjunctive intervention in the treatment of childhood ALRI was its known effect in the management of acute diarrhea. Serum zinc concentrations increase after just a few days of treatment [81, 82], suggesting that it is plausible that clinical benefits could result from rapid improvement of systemic nutritional status. However, animal data have suggested that although zinc depletion increases susceptibility to invasive bacterial infection [68], abrupt improvements in zinc availability may not enhance pathogen clearance [83]. It is also possible that the zinc supplement could have an anti-inflammatory effect that is beneficial in the acute illness.

A 2009 systematic review considered data from five trials of therapeutic zinc for the treatment of ALRI in children or adolescents, all but one of which were conducted in India or Bangladesh [50]. Only one of the five trials showed that 20 mg zinc per day shortened the duration of symptoms and an average reduction in the duration of hospitalization of about one day among children with severe ALRI (hazard ratio 0.75; 95% CI, 0.57 to 0.99) [84]. A second trial showed a benefit in boys but not girls [82]. The other three trials did not find significant benefits of zinc; in fact, a trial involving indigenous children in Australia found that zinc supplements increased the rate of readmission to hospital [85].

Two trials published in 2010, and thus not included in the 2009 review, reinforced the apparent lack of overall therapeutic effect across diverse settings in South Asia. In Nepalese infants and young children aged 2 to 35 months with severe ($n=149$) or non-severe ALRI ($n=2479$) in a population known to be zinc deficient, adjunctive treatment with 10–20 mg zinc daily for 14 days did not reduce the risk of a change in antibiotics or hospital admission, or affect the time to resolution of signs and symptoms, regardless of age or disease severity [86]. In India, a 5-day course of 20 mg oral zinc per day did not significantly shorten the duration of hospital stay or time to resolution of signs and symptoms of severe ALRI in infants and young children aged 2 to 24 months ($N=104$) who had normal pre-treatment serum zinc concentrations [87]. However, neither of these studies was highly powered to detect subtle effects of zinc supplementation among children with severe ALRI. There are thus calls for larger trials focused on the subset of children with ALRI who are most liable to have severe bacterial pneumonia [88], but at this time there is insufficient evidence to recommend that zinc be routinely administered as adjunctive treatment for ALRI.

2.3 Prevention of The Common Cold

A few trials have considered the prophylactic effect of routine daily zinc supplementation on the incidence of upper respiratory tract infections (URIs). In a small RCT involving US air force cadets randomized to zinc or placebo for 7 months, there was no effect on the incidence of URI [89]. In a randomized trial in Dhaka,

Bangladesh, a three-month extension of adjunctive zinc treatment for acute diarrhea among children aged 6 to 23 months yielded enduring beneficial effects on subsequent diarrheal incidence, but there was no effect on the risk of upper respiratory tract infection defined by the presence of cough or sore throat [90]. However, in two separate trials of zinc administered daily throughout the winter to healthy schoolchildren in Turkey [91] and Iran [92] (15 or 10 mg/day as zinc sulphate, respectively), zinc significantly reduced both the average number of colds (defined by the presence of at least two symptoms/signs of URI) and school absenteeism. In the Iranian trial, zinc also led to significantly fewer antibiotic prescriptions [92]. These studies were the only two preventive trials included in a recent Cochrane meta-analysis [93]. Larger confirmatory studies using specific and objective URI case definitions are warranted.

2.4 Treatment of The Common Cold

The use of zinc administered as lozenges or in other oral/nasal vehicles to treat the common cold remains as controversial now as it was when George Eby and colleagues first advanced the idea in the early 1980s [94]. In support of several narrative reviews[95-97] but in contrast to the conclusions of previous meta-analyses[98, 99], a recent Cochrane Collaboration review concluded that zinc lozenges or syrup administered within one day of onset of cold symptoms significantly reduced the duration and severity of illness[93]. There was substantial heterogeneity among the six trials included in the meta-analysis, but the authors noted that there were too few trials to conduct sub-group analyses. The overall amount of available data was surprisingly meager – in fact, there were fewer than 800 participants in total in the pooled analyses.

In an audit of the literature published in 2007, Caruso et al. highlighted the methodological limitations of many studies in this field, and deemed a minority of clinical trials of zinc lozenges, nasal sprays, or nasal gels to be sufficiently rigorous for guiding practice recommendations [100]. Eby (2010) has contended that variations in the findings among clinical trials can be explained by differences in the ionic zinc (iZn) content of the lozenges studied, because many zinc lozenges contain zinc-binding ligands (e.g., citrate) that are used to mask the astringent metallic taste[101]. Positively-charged iZn, rather than protein-bound zinc, is believed to be responsible for zinc's immunomodulatory and anti-viral properties. Eby found that the magnitude of the treatment effect (e.g., reduction in duration of symptoms) strongly correlated with the lozenge iZn content and estimated total daily iZn intake prescribed, but not with the total amount of (iZn plus bound) zinc[101].

In summary, existing evidence suggests zinc lozenges with high ionic zinc content (e.g., zinc acetate) and adequate dosing frequency may have beneficial effects for the treatment of the common cold in adults. However, given the conflicting literature, the burdensome treatment regimen, and unresolved safety issues, confirmatory trials of zinc acetate in adults and children are needed before zinc lozenges can be widely recommended. Ideally, studies should be designed and adequately powered to assess the effect on URI complications and secondary bacterial infections.

It is important to note that despite potential efficacy[102], there are concerns that the intranasal application of zinc compounds may cause anosmia[101]. For this reason, the Food and Drug Administration in the US recommended in June 2009 that consumers discontinue the use of zinc gluconate gel products marketed for the treatment of the common cold.

2.5 Other Respiratory Tract Infections

There has been limited study of the prophylactic or therapeutic effect of zinc supplementation for respiratory infections other than childhood ALRI or the common cold. A Cochrane review found scant and conflicting evidence related to the effect of zinc on the prevention of acute otitis media in children [103]. There is also emerging interest in the potential role of zinc in the prevention of seasonal or pandemic influenza [104], but clinical studies have not yet been undertaken. Preliminary trials have suggested that routine zinc supplementation may reduce the risk of ALRI among patients with conditions that place them at high risk of chronic or acute lower respiratory infection, such as sickle cell disease [105] and cystic fibrosis [106]. These results merit further study given the plausibility of the findings, particularly in patients with underlying nutritional vulnerabilities.

3. Malaria

The connection between nutrition and malaria pre-dates modern malaria prophylaxis. It has been shown that zinc deficiency may act as a risk factor for malaria infection [6, 107] and that during an acute episode of malaria, plasma zinc is reduced as part of the acute phase response to infection with *Plasmodium falciparum* [108]. However, this correlation was not observed among young adults with *Plasmodium vivax* in Turkey; [109] thus it is possible that infection with *P. vivax* illicits a different inflammatory response than what has been observed with *P. falciparum*.

3.1. Prevention

Several studies have investigated zinc supplementation for the prevention of malaria among children less than 5 years of age. In Papua New Guinea, children 6-60 mo of age who received 10 mg of zinc six times per week for 46 weeks had 38% fewer slide confirmed malaria episodes (*P. falciparum*) reported at health centers than children who received placebo. This benefit of zinc was more pronounced when looking specifically at episodes with heavy parasitemia ($\geq 100,000 \mu\text{L}$) [110]. In holoendemic Burkina Faso, children receiving daily zinc (10 mg) and vitamin A demonstrated an overall reduction in the number of malaria episodes and a delay in the time to first episode when compared to children who received only vitamin A supplementation [111]. Bates et al also observed a trend toward fewer malaria clinic visits among children in The Gambia who were supplemented with zinc compared to children who received placebo [112]. Muller et al observed no effect of daily zinc supplementation (12.5 mg) on any malaria clinical outcome when compared to children receiving placebo [113]. Prevention of *P. vivax* was studied in a trial among children 6 mo to 15 years of age and though there was a trend in lower malaria morbidity specifically among children less than 5 years of age, this was not statistically significant [75]. While the benefits of routine zinc supplementation appear to be inconsistent, with regard to a specific reduction in malaria incidence, the provision of daily zinc, especially among high-risk groups, could potentially result in a decrease in overall child mortality in areas where malaria is holoendemic [56, 76, 114].

3.2. Treatment

There has been one study thus far assessing the effect of zinc as an adjunct treatment for malaria [115]. In this multi-center study, 1087 children with slide confirmed *P. falciparum* malaria in Ghana, Tanzania, Uganda, Ecuador, and Zambia were randomized to receive zinc (20-40 mg/day) or placebo. There was no effect of zinc on any clinical outcome.

4. Measles

The treatment of measles with zinc was assessed in one study enrolling hospitalized children 9 mo to 15 years of age in India. While more than half of children with measles had low plasma zinc, zinc supplementation had no effect on time to recovery or on the proportion cured 6 days after treatment [116].

5. Tuberculosis

Low serum zinc has been observed among children and adults with tuberculosis at varying stages of disease [117, 118]. Zinc status appears to be directly correlated to disease progression in that patients with more advanced disease have progressively poorer serum zinc status; yet, this can be improved with anti-tuberculosis therapy, [118, 119] and it has been suggested that increasing plasma zinc status could be used as a proxy for response to therapy [118]. Despite these observed correlations, the few randomized placebo controlled trials of zinc alone that have been conducted to date have failed to show any effect of supplementation on clinical tuberculosis outcomes [120, 121].

6. HIV/AIDS

Human immunodeficiency virus (HIV) is a complex disease resulting in increased risk for opportunistic infections and micronutrient deficiencies [122]. Zinc is critical for proper immune function, yet is also a key component of viral replication, and thus its role among HIV-positive individuals is important to understand [4, 122]. Zinc deficiency has been well documented among HIV-infected persons across all socio-economic strata as a result of decreased food intake and increased stool output from frequent diarrhea episodes [123-125]. The relationship between plasma zinc and immunologic or functional outcomes is not as clear. Some have found a relationship between low plasma zinc and compromised immune function [126] resulting in fewer diarrhea episodes and delayed immunologic failure as a result of supplementation [127, 128]. However, among other populations, such as HIV-positive pregnant women, zinc supplementation has not been shown to have an effect on clinical or functional outcomes of the mother or newborn [129, 130]. While the link between serum zinc deficiency and HIV is well established, the clinical significance of this association has yet to be determined and additional studies are needed before routine dietary or supplementation guidelines are adjusted for this population.

7. Interventions and Programs

Zinc for the treatment of diarrhea among young children has been a World Health Organization and UNICEF recommendation since 2004 [28]. Given the bounty of evidence supporting the use of zinc and the burden of diarrhea globally among children under 5 years of age, it is unfortunate that zinc for diarrhea treatment has been slow to move from science to programs around the world [131]. Diarrhea treatment includes oral rehydration salts (ORS), zinc supplementation, and continued feeding yet even the delivery of this simple package is challenging in some settings. While evidence to date suggests that high coverage rates can be achieved with targeted efforts, many countries lack the funds to support the efforts needed to enhance scale-up such as local demonstration projects to test improved delivery strategies, procurement of seed supplies for zinc, and routine monitoring to identify and overcome delivery challenges [131, 132]. There are currently no global recommendations for the use of zinc as a treatment for any other childhood disease.

While there is widespread evidence suggesting that preventive zinc supplementation would improve zinc status leading to fewer illnesses and improved growth among young children in many developing countries [133], there are no formal recommendations for routine supplementation programs at this time. Zinc must be provided on a routine basis, thus cannot be delivered in a twice-yearly supplement as is done with vitamin A supplementation programs. In addition, some evidence suggests that zinc competes with other micronutrients, specifically iron, and thus providing both zinc and iron together will minimize the beneficial effect observed when either nutrient is provided alone [76]. While trials thus far have focused on the effect of zinc supplementation, alternatives such as micronutrient sprinkles and food fortification may also prove to be as effective. Though there are data to suggest that when zinc is delivered in the form of sprinkles or a fortified food, individual plasma zinc rises in some but not all studies, evidence is lacking that these approaches result in improvements in functional outcomes such as reductions in incident disease and improved growth [76]. There are currently few examples of health or nutrition programs that successfully reach preschool aged children to provide a daily, routine intervention for long periods of time, thus more research is needed to develop effective delivery strategies to ensure zinc reaches the children most in need.

8. Conclusion and Perspectives

Zinc is critical for immune function and thus adequate intake of zinc is important for the prevention of and recovery from disease. The possible link between infection and zinc deficiency has been shown across several infectious diseases including diarrhea, pneumonia, malaria, HIV, and tuberculosis. In populations where dietary zinc is inadequate, it is clear that zinc deficiency increases susceptibility for infection and makes it more difficult to fight illnesses. However, less certain is the role of zinc supplementation for the prevention and treatment of these diseases. The data supporting zinc for diarrhea prevention and treatment are robust for children under 5 years of age, yet the evidence is less clear for other diseases. Providing additional zinc via supplementation may be an important adjunct therapy for other infectious diseases especially for severe disease or among individuals at risk of zinc deficiency. Unfortunately, subclinical zinc deficiency can be difficult to detect on an individual

basis further complicating a full understanding of this complex nutrient – infection relationship. Continued research is needed to further define the role of zinc in preventing and treating infectious diseases. Providing zinc to patients in need may offer an inexpensive and safe alternative for improving response to treatment and in turn enhancing overall health.

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12. Zinc in Critical Illness and Sepsis

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Abstract. Zinc availability affects the host response to major trauma and infection and is associated with the classic acute phase response. During the acute phase response plasma zinc levels precipitously decline as a result of mobilization of zinc into the intracellular compartment to assist with vital metabolic functions that enable immune defense, tissue repair and recovery. Despite knowing some but not all of the essential roles that zinc plays in host defense, little is known regarding metabolic zinc requirements at the onset of and during the acute phase response in the context of critical illness. Recent evidence suggests that zinc supplementation may have a beneficial role in the critically ill and that zinc deficiency, prior to host insult, may be disadvantageous. In this review, new data on zinc metabolism, its implication in the pathogenesis of critical illness with a focus on sepsis, and its therapeutic effects are summarized.

Keywords: critical illness, sepsis, supplementation, zinc, zinc deficiency

Introduction

Critical illness is defined as a disease or state in which death is possible or imminent. During the early stages of critical illness, typically the consequence of infection or substantial tissue injury, the acute phase response is activated. The acute phase response is a beneficial and natural systemic response that is designed to restore order following a state of physiologic disarray [1]. During the activation stage a local response at the site of injury occurs characterized by but not limited to platelet aggregation, clot formation, vasodilation, and recruitment of granulocytes and mononuclear cells. This leads to the rapid production and release of cytokines, chemokines, and other soluble mediators that distribute throughout the body and invoke a systemic reaction characterized by fever, leukocytosis, gluconeogenesis, and alterations in acute phase proteins, principally within the liver [2]. It is well established that a decrease in circulating levels of iron and zinc [Zn] occur during the systemic response to infection or inflammation. Whereas hypoferremia is a strategic maneuver that sequesters bioavailable iron from pathogenic microorganisms [3], a beneficial corresponding role of hypozincemia has not yet been established. Given the many vital biological roles that zinc plays, it has been postulated that mobilization of zinc from the vasculature into the cellular compartment is an essential component of the initial host response that facilitates proper cell signaling, gene transcription, protein production, protein function, and organelle function [4]. Further, zinc assists in regulation of intracellular redox balance and therefore may play a role in cytoprotection and

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resistance to microbial invasion. For these reasons, it is predicted that zinc nutrition and metabolism before and at the time of critical illness has significant influence on the overall host response to injury or infection both in terms of susceptibility and recovery.

1. Nutrition, Zinc, and Critical Illness

Over the past 40 years advances in enteral and parenteral nutrition formulations have made the provision of nutritional support possible for most critically ill patients. Specialized formulations and additives that provide specific micronutrients are now available. Although nutrition supplementation in intensive care units (ICUs) is widespread and considered common practice, many aspects of nutrition support remain controversial. This is in part due to a paucity of large, well-controlled, randomized clinical trials. While it is assumed that nutrient depletion can increase morbidity and mortality, and that correction or prevention of such nutrient depletion is beneficial, it has been difficult to isolate the independent contributions of individual micronutrients in critically ill subjects with respect to patient outcome. In large part, this is the consequence of an unpredictable disease onset within a population that possesses heterogeneous disease processes across spectrums of disease severity. In other words, there are many independent variables that cannot easily be controlled for during the design of a clinical trial. Further, many of the most current published clinical trials have studied combinations products thereby making it impossible to quantify the contributions of any one ingredient to patient outcome.

It is well established that adaptive changes occur as part of the stress response to overwhelming injury in order to conserve body composition while at the same time mounting a robust response to fight infection and initiate healing. Critically ill subjects are highly vulnerable to malnutrition due to maladaptation of body composition particularly if macro- or micronutrient intake is sub-optimal for sustained periods of time. Sustained nutritional deficits during the recovery phase by neglecting to sustain macro- or micronutrient intake has the potential to exacerbate biochemical abnormalities and perpetuate organ dysfunction thereby adversely impacting patient outcome during critical illness. For these reasons nutrition supplementation, in the form of enteral nutrition, is preferred and often initiated within the first 24 hours of ICU admission unless the risk of doing so significantly outweighs the proposed benefit [5, 6]. In fact, when nutritional support is administered to critically ill patients in the catabolic phase of disease it has been shown to reduce ICU days, the incidence of infection, and improve patient prognosis. However, to date, conventional practice has focused primarily on replenishing macronutrients, in terms of optimizing caloric and protein requirements. Attention to micronutrients including vitamins and trace elements is often neglected, with the noted exception of burn patients, although recommendations are now emerging in which the use of immune-modulating formulations are advocated in critically ill adult surgical and medical patients [7].

Assessment of the state of nutrition, or more accurately the level of nutritional deficit, would appear to be a logical first step in determining patient-specific requirements. However, this is very difficult to do in critically ill patients due to confounding factors including but not limited to edema, changes in plasma protein binding, alteration in hemodynamics, and alterations in metabolism. Taking this into consideration, five essential trace elements are approved by the United States Food and Drug Administration (FDA) for parenteral use and include zinc, selenium, copper,

chromium, and manganese. Of these, zinc is the most abundant in the body. Typical of most trace elements, accurate measurement of plasma zinc levels and hence the ability to determine zinc status in critically ill subjects is complicated [8]. Collection using special trace-element-free equipment into special tubes is needed. Specimens often need to be sent to specialized reference laboratories thereby delaying acquisition of results. Additionally, as previously identified, serum levels decrease in response to systemic inflammation thereby potentially leading to false interpretation of findings. Analysis of zinc-binding proteins such as tissue metallothionein levels, have been attempted as a surrogate marker in this setting but have largely proven to be inaccurate [9]. More precise measures of zinc status that include kinetic studies using isotopes such as ^{65}Zn are not practical in the critical care setting. However, despite these limitations, serum Zn levels, although representing only 0.1% of total body Zn, is considered the best clinical test available. Currently, established recommendations for zinc supplementation do not exist for the critically ill. Whether zinc supplementation or pharmacologic treatment is justified will be taken into consideration later in this chapter.

2. Sepsis and Zinc Metabolism

The systemic inflammatory response syndrome (SIRS) occurs as a consequence of critical illness. When critically ill subjects meet criteria for SIRS, in the context of infection, they are said to have sepsis. Critically ill patients with sepsis, who progress to the development of cardiovascular and other organ failure, are said to have septic shock or severe sepsis respectively. Although sepsis and septic shock are highly heterogeneous clinical syndromes, the recognition of sepsis and septic shock drive clinical decision processes and dictate specific therapeutic strategies. Importantly, the mortality associated with sepsis and septic shock remains unacceptably high [10]. Sepsis is a frequently occurring serious medical condition caused by infection leading to systemic activation of the host inflammatory response and tissue injury. Subsequent failure of vital organs is the leading cause of morbidity and mortality in sepsis patients [11]. In the past decade it has become clear that the mortality associated with septic shock is strongly associated with over-activation of the inflammatory response [12, 13]. Increased expression of NF- κ B-mediated cytokines including interleukin (IL)-1 β , tumor necrosis factor (TNF) α , and IL-6 has been reported to be associated with an increased risk of vital organ failure in sepsis along with a worse prognosis [14]. With respect to zinc metabolism, human and animal studies have demonstrated that plasma zinc concentrations rapidly decline at the onset of sepsis as a result of the acute phase response due to redistribution of zinc into the cellular compartment without obvious body losses [15, 16]. This would suggest that zinc redistribution and factors that control zinc metabolism, such as zinc transporters, are crucial during the early stages of the host response against systemic infection. While a majority of zinc redistribution is accounted for by the liver, it is now clear that this phenomenon is not restricted to the liver and occurs throughout the body in other organs and cell types.

2.1. Zinc Deficiency Increases Morbidity and Mortality in Animal Models of Sepsis

Given the many complexities associated with conducting experimental trials in the critically ill population, our group and others have resorted to small animal models of

sepsis as a first step toward better understanding zinc metabolism. Using an animal model of polymicrobial sepsis that involves cecal ligation and puncture (CLP), we observed that moderate zinc deficiency induced by dietary restriction, as determined by a 2.5-fold reduction in circulating plasma zinc concentration and commensurate decrease in tissue metallothionein levels, significantly increased systemic bacterial burden within hours of infection leading to a substantial increase in morbidity and mortality [17]. The increase in mortality, which was 30% in zinc sufficient animals compared to 90% in zinc deficient animals, was remarkable considering that all physical attributes of the animals prior to sepsis, including weight, appearance, and activity, were identical. We also observed that short-term zinc supplementation in deficient animals prior to sepsis significantly improved animal survival although it did not fully return to that of the zinc sufficient control group. These findings are consistent with previous animal studies demonstrating that zinc deficiency increased shock and liver injury following endotoxin administration and that zinc supplementation abrogates NF- κ B activation in an endotoxin-induced inflammation model in mice, thereby reducing TNF α production and subsequent liver injury [18]. Although these studies identify an important role for zinc during the host response to systemic infection, they did not determine whether zinc replacement after the onset of infection improves outcomes, a topic that will be discussed later in this chapter.

2.2. Zinc Deficiency and Infection in Humans

Remarkably, the features of zinc-mediated immune dysfunction that were observed in animal models of sepsis, characterized by increased apoptotic splenocytes and augmentation of the initial innate immune response, were remarkably similar to the features initially described by Hotchkiss and colleagues in human sepsis non-survivors [14, 19]. These observations also parallel human studies demonstrating that human sepsis non-survivors can be distinguished from sepsis survivors by exaggeration of their pro-inflammatory response [12]. Taken together, these findings provide supportive evidence that zinc deficiency and perturbations in zinc metabolism have the potential to contribute to and intensify the initial immune response in the setting of infection thereby predisposing the host to worse outcomes. As discussed in chapter 11, this is consistent with human studies demonstrating that zinc deficiency is a significant world health problem that contributes to approximately 800,000 deaths annually, particularly in developing countries, and is one of the leading causes of newly acquired infections [20-25]. Most of these infections originate in subjects at mucosal surfaces, such as the gastrointestinal or respiratory tract, following invasion of bacteria that, under normal circumstances, should not readily penetrate through the front-line defense of the innate immune system. Importantly, oral zinc supplementation regimens have been shown to reduce the incidence of infection in the ambulatory setting. In light of these observations, it again raises the question as to whether zinc supplementation strategies in the ICU are warranted in an attempt to reduce the morbidity and mortality associated with sepsis. Further, few if any formal studies have been conducted that quantify the incidence of zinc deficiency in the critically ill population particularly before the onset of disease. That being said, zinc deficiency in developed countries such as the United States is underestimated and predicted to affect millions [26]. Adult populations in the U.S. that are most prone to zinc deficiency include the elderly, patients with chronic illness, and alcoholics. Perhaps not coincidental, these adult populations also constitute the most vulnerable patient types for developing sepsis.

Based on this, it is clear that more studies are required in relevant animal models and more prospective clinical trials conducted in the critically ill to better understand the impact of zinc deficiency and zinc metabolism on disease susceptibility and sepsis outcomes.

2.3. Alteration of Zinc Metabolism During Sepsis in Humans

When humans are administered a sublethal concentration of endotoxin sufficient to induce the acute phase response a precipitous decrease in plasma zinc levels occur [16]. Consistent with this, Wong and colleagues were the first to observe that lower serum zinc levels and genome-level perturbations in zinc-related proteins occur more frequently in pediatric sepsis non-survivors when compared with survivors suggesting that dysregulation of zinc homeostasis negatively impacts the host response to sepsis [27, 28]. Importantly, perturbations in genes associated with zinc metabolism and adaptive immunity were more substantially depressed in pediatric patients suffering from septic shock when compared to subjects with SIRS or sepsis. Alteration of these parameters in the septic shock cohort occurred simultaneously with persistent, exaggerated expression of genes associated with inflammation and innate immunity. Gene expression patterns between the SIRS, sepsis, and septic shock patients were essentially identical during the first day of illness but then began to diverge later in the course of infection (Day 3 and beyond) [29]. Taken together these findings, that are consistent with other human trials previously identified, suggest that septic shock occurs as a consequence of over activation of the inflammatory response and may be driven in part by dysregulation in zinc metabolism. Further, the fact that SIRS, sepsis, and septic shock patient's gene expression profiles were identical within the first 24 hours of disease onset further emphasize the difficulty clinicians are faced with when trying to predict patient outcomes and personalize treatment strategies. Genome-level perturbations in zinc associated proteins in humans provides provocative new evidence that a single micronutrient, or possibly a lack thereof, has the capacity to create an indelible biological footprint that alters the host response to an overwhelming infection. Whether the footprint can be altered after sepsis onset, presumably by supplementation strategies, was not evaluated in these studies. Further, the investigators did not establish whether zinc deficiency was present in subjects before the onset of critical illness. This raises a critical question as to whether zinc deficiency, in and of itself, is a major risk factor for developing infection and sepsis, particularly in the presence of other comorbid conditions.

In an observational pilot study recently conducted by our laboratory, we compared changes in zinc metabolism at the onset of critical illness between infected (septic) and non-infected adult subjects. Subjects were evaluated within 24 hours of sepsis onset or ICU admission respectively. Consistent with past investigations, we observed that plasma zinc concentrations were below normal in critically ill, non-infected patients but further reduced in the septic (infected) cohort. Plasma cytokine concentrations were highest in subjects with lower plasma zinc concentrations. Alteration of zinc metabolism, as measured by lower zinc plasma concentrations and higher gene expression of the zinc transporter Zip8 in monocytes, was more pronounced in septic patients and correlated with increased severity of illness including cardiovascular dysfunction. Strikingly, Zip8 was the only zinc transporter out of 14 Zip and 10 Znt family members to consistently exhibit elevated message RNA levels in monocytes. Similar to past studies, we were not able to determine patient nutrition status prior to

the ICU visit so it is not clear what role if any, zinc played in exacerbating immune dysfunction and patient outcome (*In Press*). In a similar study, Cander and colleagues reported a reduction in plasma zinc levels in critically ill patients although zinc levels were not prognostic in predicting disease severity or mortality [30]. In contrast to our investigation, a distinction between infected and non-infected subjects was not made.

3. Zinc Metabolism, Sepsis, and Immune Function

Maintenance of total body zinc composition and cellular content in humans, defined as zinc homeostasis, is tightly controlled with approximately 1% of total body zinc content replenished daily by dietary intake [31, 32]. Briefly, metabolism is tightly regulated in mammals by zinc transporters, a family of multiple transmembrane domain spanning proteins that are encoded by two solute-linked carrier (SLC) gene families: *SLC30* (ZnT) and *SLC39* (Zip) as reviewed in Chapter 8. A total of 10 *SLC30* and 14 *SLC39* family members [4, 33, 34] for a total of 24 known zinc transporters have been identified in mammals. Homology between mice and humans is remarkably similar. In general, ZnT and Zip family members have opposite roles in regulating cellular zinc metabolism. ZnT transporters reduce cytosolic zinc bioavailability by promoting zinc efflux in conditions of excess, while Zip transporters function by increasing cytosolic zinc during deficient states. Given the redundancy of transporter function with respect to zinc metabolism, it is believed that zinc transporters work in concert to maintain critical intracellular zinc levels throughout the body at all times. Members of both families exhibit tissue specific expression and possess differential responsiveness to dietary zinc but also physiologic stimuli including cytokines and hormones [35]. Therefore the role of zinc transporters is absolutely critical when considering that zinc is involved in many critical cellular functions that include but are not limited to maintaining normal cellular physiology, growth, repair, and gene expression.

3.1. Zinc Transporters, Cellular Defense and Immune Function

Regulation of the host immune response by zinc is inextricably linked to zinc transporters. Although this distinction is quite obvious, only recently have discoveries begun to emerge demonstrating how this takes place. Kitamura and colleagues first revealed a direct connection between zinc metabolism and pathogen recognition [36]. Knowing that zinc deficiency adversely affects adaptive immune function, dendritic cells were challenged with the Toll-like receptor (TLR) agonist lipopolysaccharide (LPS) and a decrease in Zip6 and cellular zinc content was observed. Importantly, Zip6-mediated changes in zinc metabolism were required to coordinate the expression of major histocompatibility class II and co-stimulatory molecules in the context of antigen presentation. Zinc supplementation or over-expression of Zip6 reversed these effects. Whether this relates directly to sepsis and critical illness remains to be determined but it does provide some of the first mechanistic insight that directly connects a zinc transporter to regulation of the host adaptive immune response and demonstrates that balance between zinc metabolism and immune function is required for proper host function. In another study, Liuzzi and Cousins using both *in vivo* and *in vitro* models demonstrated that Zip14 expression is up-regulated through an IL-6-dependent manner at the onset of the acute phase response, and that this zinc

transporter plays a major role in the mechanism responsible for hypozincemia that accompanies systemic inflammation and infection[37]. Importantly, induction of Zip14 expression resulted in mobilization of zinc into the cytosol of hepatocytes, presumably to facilitate cellular defense mechanisms. In related studies, our laboratory went on to demonstrate that Zip8, the closest homologue to Zip14, was the only zinc transporter (out of 24 evaluated) to be induced in lung epithelia in response to TNF α [38]. Importantly, when the ability to induce Zip8 protein expression was compromised a decrease in cytosolic zinc in response to TNF α was observed causing Zip8 deficient cells to be more susceptible to programmed cell death. Our findings were similar to those of Begum and colleagues who first discovered Zip8, at that time identified as BIGM103, and reported induction of BIGM103 expression in response to bacterial byproducts or TNF α in primary human monocytes, although the importance of Zip8 induction was not further studied [39].

Based upon these findings, we further hypothesized that Zip8 may be a vital component of the innate immune response and questioned how and why gene expression is triggered in response to infection, especially considering that most other zinc transporters are not. As a subtle clue, we first observed that the closest neighbor to Zip8 on chromosome 4 (location 4q24) in humans is NF- κ B1, a protein that is co-translationally processed thereby liberating a 50 kDa protein (p50) that is a DNA binding subunit of the NF- κ B protein complex. Moving forward, we cloned the human Zip8 promoter region and revealed an NF- κ B binding domain (unpublished observation). Perhaps most important, we have recently discovered that induction of Zip8 at the onset of the NF- κ B-mediated innate immune response is essential and helps to coordinate the extent of immune activation, in a zinc dependent manner, thereby potentially having broad implications with regard to how micronutrient metabolism impacts host defense. Whereas Zip14 is critical in coordinating zinc metabolism in the liver, Zip8 appears to provide similar function in monocytes, macrophages, and other tissues such as the lung. Therefore, these two transporters and perhaps others yet to be identified, work coordinately to guide elements of zinc metabolism at the onset of a systemic insult to bolster defense mechanisms. Clearly, the ability to rapidly mobilize zinc into the cell is critical in maintaining cell function and viability in the setting of a hostile, inflammatory environment as depicted in Figure 1[31].

Collectively, this demonstrates that certain zinc transporters are designated more so for homeostatic function that is directly linked to dietary intakes and maintenance of adequate intracellular pools while others, such as Zip6, Zip8 and Zip14, have evolved to serve specialized functions that coordinate host defense. At the cellular level, deficits in zinc content are not readily apparent but do lead to alteration of zinc transporter expression in a compensatory manner in order to maintain cellular homeostasis. In contrast, deficits in intracellular zinc content becomes readily apparent upon provocation, perhaps as the result of an acquired infection, resulting in diminished cytoprotection, wound healing, and tissue repair. For example, our group and others have shown that deficits in the available intracellular zinc pool enhances apoptosis in response to inflammation in part through overproduction of reactive oxygen species (ROS) [40-44]. With respect to immune function, it is well established that zinc deficiency rapidly diminishes antibody- and cell-mediated responses in both humans and animals resulting in increased risk of infection [39, 45]. In particular, thymic atrophy, lymphopenia, and compromised cell- and antibody-mediated responses are immunologic hallmarks of zinc deficiency in humans [46]. Whereas, a substantial body of evidence exists describing the impact of zinc deficiency on adaptive immune

function [45], less is known regarding the influence on innate immune function. Clearly more studies are necessary to help us better understand the important relationship between zinc metabolism and the host response to critical illness and overwhelming infection. Completion of such studies would enhance our understanding of the pathogenic mechanisms that account for zinc deficiency in the setting of infection and potentially reveal novel biomarkers that would enhance our ability to predict disease susceptibility patterns.

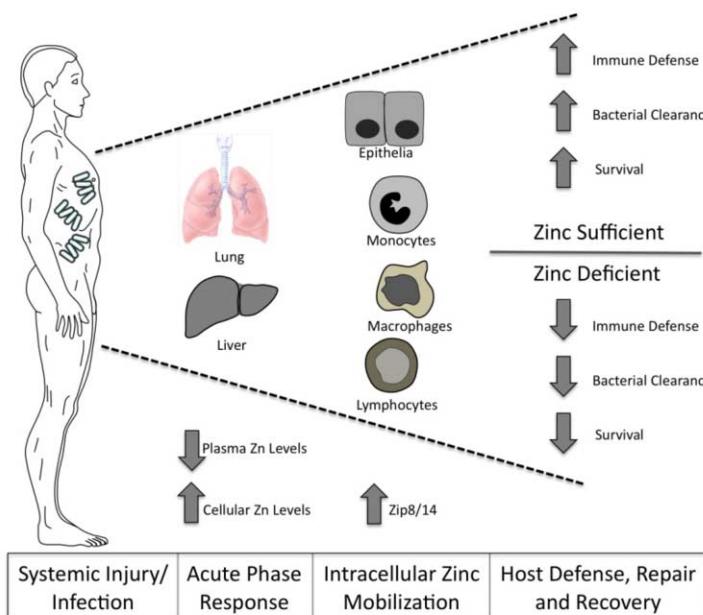


Figure 1. At the onset of systemic injury and infection the acute phase response is activated leading to the mobilization of zinc from the blood compartment into tissues and cells. The transport of zinc into the cell is facilitated by the zinc transporters Zip8 and Zip14 whose expression is induced by cytokines. These alterations in zinc metabolism are critical in guiding the initial host response that influence both innate and adaptive immune function. If the host is zinc sufficient, the chance of recovery is improved whereas zinc deficiency increases morbidity and mortality.

3.2 Zinc, Cellular Defense, and Signal Transduction

The cellular consequences of zinc deficiency manifest through oxidant stress, modulation of inflammation, and in extreme situations, premature cell death. Knowing that many proteins require zinc for proper function (greater than 3% of the human genome) it is plausible to consider that signal transduction pathways would be influenced by zinc metabolism in the setting of sepsis and indeed this has been shown previously [47] (see Chapter 6). Nuclear Factor (NF)- κ B is a potent transcription factor central to many of the signaling networks involved in the host response to critical illness and sepsis [48]. NF- κ B is activated by most pathogens commonly associated with sepsis and its activity is markedly elevated throughout the body of sepsis patients [13]. Moreover, higher and prolonged activation are associated with a pronounced pro-inflammatory response and higher mortality rates in sepsis patients

[12]. Relative to zinc metabolism and sepsis, NF-κB activation is directly linked to superoxide dismutase (SOD) function and formation of reactive oxygen species (ROS), both a driving force and byproduct of inflammation. Previous reports including our own [17] demonstrate that zinc deficiency leads to attenuation of SOD activity thereby favoring an increase in the formation of ROS that leads to irreversible detrimental biochemical effects within the cell. In fact, zinc itself has the potential to act indirectly as an antioxidant by virtue of its interaction with sulfur (Zn-S). Within the cell the reversible Zn-S interaction regulates mechanisms of enzyme catalysis, allows zinc to be tightly bound and yet to be available, and, importantly, generates redox-active coordination environments for the redox-inert zinc ion thereby allowing zinc to assist in “buffering” oxidant environments (see chapter 4) [49]. Therefore, zinc deficiency decreases the intracellular capacity to tolerate highly oxidant environments as would occur in the setting of sepsis. Relative to NF-κB, the function of zinc as an immunomodulator is well established but controversial since it has been identified as both an activator [49-52] and repressor [18, 53, 54] of this pathway. Knowing that these investigations utilized *in vitro* models with contradictory findings, our group took an alternative approach and again, evaluated a small animal model of sepsis. Taking advantage of BALB/c NF-κB luciferase mice in which luciferase expression is under control of the NF-κB promoter, we were able to unequivocally demonstrate that nutritional zinc deficiency resulted in a substantial increase in the rate and extent of NF-κB activation systemically including all vital organs studied in response to systemic polymicrobial infection [55]. Further, short-term oral zinc repletion prior to sepsis onset reduced NF-κB-dependent luminescence in all vital organs. Increased NF-κB activation in this zinc deficient animal model coincided with a marked increase in the circulating levels of NF-κB-dependent cytokines and chemokines and a significant increase in mortality whereas, zinc supplementation reduced circulating cytokine levels to that of zinc sufficient mice. It is important to point out that alterations in innate immune activation occurred without differences in Toll-like receptor (TLR) expression, suggesting that changes were not a consequence of perturbations in pathogen recognition upstream of NF-κB activation (unpublished observation). These findings demonstrate that zinc deficiency is a predisposition to worse outcomes in the setting of critical illness through perturbation of a critical signal transduction pathway. Based on extensive studies demonstrating the impact of zinc on other signal transduction pathways (see Chapter 6), it is predicted that zinc deficiency imparts many effects during critical illness through multiple signaling networks, although this remains to be determined.

4. Practical Considerations for Zinc Replacement

Over the past four decades a number of studies have been conducted in animal models that evaluate the capacity of zinc to improve survival in the setting of lethal bacterial infection. In general, studies involved adult mice or rats that received lethal injections of different strains of *Salmonella*, *Escherichia coli*, or LPS [18, 56-61]. Additionally, animals received zinc via systemic injection either before, during, or after the challenge with the primary endpoint determinant being survival. Interestingly, the doses of zinc administered varied greatly (range of 3 to 17.6 µg elemental zinc/gm body weight) when compared equally on a microgram of zinc per gram of body weight basis across

all studies. Further, different salt forms (chloride, acetate, sulphate, or gluconate) were utilized thereby making it difficult to compare study findings. Perhaps not surprising, there was significant disparity between individual study results. In general, zinc was most effective at improving animal survival when administered before or at the time of bacterial challenge. In most studies, the animals were zinc sufficient at the time of infection therefore the contributions of zinc deficiency were not evaluated. Unfortunately, most studies did not evaluate the impact of zinc as a post infection intervention, which would be a superior approach in terms of clinical relevance. In the one study that did, the beneficial effects of zinc supplementation diminished the longer zinc administration was delayed suggesting that a short "window of opportunity" may exist during the earlier stages of disease. Relative to the ICU setting, these studies perhaps raise more questions than they answer. Zinc treatment in these trials utilized a different salt forms and a broad range of doses that by definition would be considered to cover a broad spectrum of supplemental to supraphysiologic. As is the case with critically ill patients, it is not plausible to intervene with rapid zinc supplementation before disease onset so the clinical utility of these findings is obsolete. Further, in the ICU setting, enteral feeding is commonly preferred in place of total parenteral nutrition so that the gastrointestinal tract can remain operational during patient recovery. Little if anything is currently known regarding zinc absorption from the gut during systemic illness. While it is plausible that parenteral administration assures one hundred percent bioavailability of zinc into the systemic compartment, as identified previously, enteral feeding is preferred in the critically patient[7]. If the benefits of zinc administration in the critically ill is to be realized, clearly more animal studies will need to be conducted in relevant disease models that more closely resemble the patient care setting in the ICU environment.

Over the past decade, nutritional supplementation of the critically ill has shifted. Whereas nutrition support teams previously focused on minimizing the loss of calories and proteins, the most current treatment strategies have utilized pharmaconutrition in which nutrients are strategically provided to patients in order to change the course of inflammatory, immunologic or metabolic pathophysiologic imbalances. Successful examples demonstrating improved patient outcomes using micronutrients such as arginine, glutamine, and antioxidants has begun to emerge. As one example, it is now known that critically ill subjects often have depletion of antioxidant stores and a compromised ability to prevent oxidant induced damage [62]. This is important because increased free radical generation leads to augmentation of the inflammatory response, increased cell injury, and increased morbidity and mortality in the critically ill [63]. Zinc has been utilized as one of several ingredients in antioxidant cocktails that have been shown to improve survival in critically ill patients. However, because multiple additives were administered to these subjects, it has been difficult to determine the relative contribution, if any, of zinc in these trials. Recently, Heyland and colleagues utilized a systematic approach to review the literature on zinc supplementation in the critically ill adult patient [64]. Strict criteria were used to identify well-conducted randomized clinical trials in subjects that by definition were mechanically ventilated and critically ill. Seven articles were originally identified that reported some form of zinc supplementation in critically ill subjects. In 3 studies, zinc was administered as part of a complex enteral feeding mixture and these studies were therefore excluded since patient outcome could not be solely attributed to zinc. Of the four trials evaluated, all involved systemic intravenous administration of zinc either alone (one trial) [65] or in combination with other antioxidants [3, 66-68]. When all

four studies were aggregated, zinc supplementation was associated with a trend toward a reduction in mortality. The authors concluded that the current evidence from randomized clinical trials, although beneficial, is too sparse to develop any conclusive recommendations about the role of high dose zinc supplementation in the critically ill. Many uncertainties remain with respect to the optimal route of administration, optimal dose, and optimal methods to determine patient benefit. To move forward, additional clinical trials will need to first be conducted that establish an optimal zinc replacement dose in the critically ill. How much zinc is required and over what time frame to restore circulating plasma levels? If this can be achieved, what monitoring parameters aside from survival should be evaluated to show improvement? Additionally, zinc administration should not impose further adverse effects thereby compromising an already fragile patient. Finally, do all critically ill patients need zinc or just patient's with documented zinc deficiency? Clearly, much work lies ahead before zinc supplementation will become common practice in the ICU setting.

5. Conclusion and Perspectives

Zinc deficiency has profound effects on host defense including both innate and adaptive immunity. Our interest has been to determine to what extent if any, alteration of the innate immune response may be the result of micronutrient deficiencies including zinc, thereby predisposing individuals to increased risk of severe infection. Based on emerging evidence, it is becoming clear that zinc sufficient individuals have a decreased risk of acquiring infection when compared to zinc deficient subjects and that zinc supplementation may provide an advantage in decreasing the severity of infection [25]. This is supported by animal studies demonstrating that zinc supplementation at the correct dose and time may decrease mortality [18,56,58]. In the setting of sepsis, perturbations in zinc homeostasis rapidly occur in humans leading to mobilization of zinc into the cellular compartment. However, the role of zinc metabolism and zinc transporter function is less clear and offers many exciting challenges and opportunities given the dynamic complexity of the host response at the onset of overwhelming infection. Based on our observations involving cell culture, small animals, and now human studies, we contend that zinc plays an important immuno-modulatory role during the early stages of host defense and critical illness. In particular, zinc deficiency leads to an increase in bacterial invasion, an increase in the initial inflammatory response and more collateral tissue damage as a consequence of dysregulated immune function. Based on recent observations utilizing an animal model of sepsis, these events significantly increase the risk of mortality. Although much remains to be known regarding the role of zinc transporters in the setting of infection, a picture has emerged which indicates that many zinc transporters are designated to maintain intracellular pools in concert with dietary intakes whereas, in sharp contrast, specific zinc transporters, including Zip6, Zip8 and Zip14, have evolved to coordinate and fine tune molecular signals that direct the host innate and adaptive immune response.

It is our intent that further studies will reveal novel insight regarding micronutrient metabolism in the context of sepsis that will improve our ability to accurately identify zinc deficiency in at risk subjects and by doing so, prevent infection or more effectively treat patients after infection has occurred.

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13. Zinc in Allergy, Autoimmune, and Hard and Connective Tissue Diseases

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Abstract. Zinc (Zn) is essential for normal cell structure and physiology. Its deficiency causes growth retardation, immunodeficiency, and neuronal degeneration. Zn homeostasis is tightly controlled through Zn transporters and metallothioneins, which regulate Zn concentration, its distribution in individual cells, and contribute to Zn signaling in cells. Zn intracellular signaling regulates immune reactions as well as hard and connective tissue development. Although many molecules involved in these processes have Zn-binding motifs, the molecular mechanisms of Zn's role have not been clarified. Recently, we and other groups demonstrated that Zn signaling plays diverse and specific roles in vivo and in vitro, in studies on the genetic knockout of Zn transporter functions. In this section, we discuss the impact of Zn signaling on the mast cell-mediated allergy response, T cell-mediated immune response, and development of hard and connective tissues. We also describe Zn signaling dysregulation as a leading health problem in allergy and immune response, and in skeletal and connective tissues' development.

Keywords. Zinc signaling, Zinc transporter, Allergy, Autoimmune, Connective Tissue, Signal transduction

Introduction

This review discusses our current understanding of Zinc (Zn) transporters and Zn signaling's roles in allergy, immune, and autoimmune disorders, and in hard and connective tissue development. The essential trace element Zn [1, 2] functions as a neurotransmitter [3, 4] and intracellular signaling molecule [5, 6]. Zn's various effects on the immune and nervous systems have been demonstrated both in vivo and in vitro; these effects mainly depend on Zn concentration [4, 7]. Many researchers have reported that Zn depletion decreases immune function. Natural killer cell-mediated cytotoxic

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activity, antibody-mediated responses, and host defenses against pathogens and tumors are reduced in Zn-deficient mice [8-10] (see chapter 10).

Zn has an essential constitutive role in the conformation and activity of many enzymes, transcription factors, signaling molecules, and other components involved in these processes. On the other hand, at high concentrations Zn can be cytotoxic and induce apoptosis in T and B cells [11, 12]. Zn's concentration and distribution in and between cells is controlled by Zn transporters [13], including the Slc39/ZIP and Slc30/ZnT families (see chapter 8), which increase and decrease intracellular Zn levels, respectively [14], and is buffered by Zn-binding molecules [15-20] (see chapter 4). Zn also acts as an intracellular signaling molecule: extracellular stimuli can affect intracellular Zn either by regulating the transcription of genes for Zn transporters and Zn-binding molecules, or transcription-independently—as in the case of the Zn wave [21] (see chapter 6). We categorize the former as late Zn signaling, and the latter as early Zn signaling [5]. While many studies have shown that Zn is important to the immune system, and that imbalances in Zn homeostasis lead to various disorders, it is not known how Zn homeostasis and signaling are regulated in immune cells or whether Zn transporters are involved in immune cell function.

We here describe how Zn, its homeostasis, and its signaling affect biological events in allergy and autoimmune functions, as well as in hard and connective tissue development.

1. Zinc's Role in Mast Cell Function and Allergic Response

Mast cells, eosinophils, and basophils are involved in allergic reactions such as anaphylaxis, asthma and atopic dermatitis [22-24]. Activated mast cells secrete two classes of mediators. The first, pre-formed mediators stored in granules, are degranulated and secreted quickly. The second class of mediators, cytokines and chemokines, must be newly synthesized and thus are secreted more slowly. These secreted molecules play leading roles in the inflammatory reactions observed in patients with allergies.

The Zn probe Zinquin has been used to determine intracellular Zn levels and distribution, and to visualize distinct Zn pools in allergy-related cells. Mast cell granules fluoresce intensely with Zinquin [25]. Airway epithelial cells are also rich in Zn [26]. Zn deficiency increases allergic eosinophilic inflammation, whereas dietary zinc supplementation reduces its intensity [27]. Interestingly, Zn deficiency is a risk factor for developing asthma [28, 29]. Also, high *SLC39A2/ZIP2* expression levels are seen in the leukocytes of asthmatic infants [30]. These reports indicate that Zn is involved in the development of allergic disease. However, the precise roles of Zn and Zn transporters in allergy-related cells are not well understood.

Zn is required for both degranulation and cytokine production in mast cells [31] (figure 1). The Zn chelator *N,N,N,N*-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) inhibits mast cell histamine release, cytokine production, and lipid mediator secretion; Zn supplementation rescues these inhibitory effects. Mast cell function is not affected by other metal chelators [31]. Similarly, Zn depletion caused by TPEN or the clinically-used heavy-metal chelator DMPS [32] inhibits the mRNA expression of chemokines such as Eotaxin in human lung cell lines [33]. These observations point to Zn chelators and their derivatives as potential antiallergic drugs that may act differently from currently available drugs, such as histamine antagonists.

Mast cell degranulation begins when high-affinity receptor for IgE (Fc ϵ RI) is aggregated with its specific antigen. It is regulated by calcium-independent, microtubule-dependent granule translocation step and calcium-dependent membrane fusion step of granule membrane with the plasma membrane [34]. TPEN suppresses Fc ϵ RI-induced granule translocation, though it has little effect on calcium mobilization or on other Fc ϵ RI functions, such as the Fc ϵ RI-induced tyrosine phosphorylation of various signaling molecules. Since granule translocation depends on cytoskeletal proteins such as tubulin and actin [35], and microtubules are critical for both granule translocation and vesicle transport [34, 36], TPEN was hypothesized to affect microtubule assembly. However, as shown by Kabu et al. TPEN does not suppress Fc ϵ RI-induced microtubule formation, suggesting that its target might be one or more Zn-regulated molecules that link directly with microtubules and granules.

Kinesin receptors are linker-cargo proteins essential for microtubule-dependent vesicle trafficking [37], and therefore TPEN's target might interact indirectly with granules via kinesin. TPEN suppresses Fc ϵ RI-mediated cytokine production as well as interleukin (IL)-6 and tumor necrosis factor (TNF) α mRNA transcription. Its stimulation activates Protein kinase C (PKC), which is involved in cytokine production through Nuclear factor-kappaB (NF- κ B) activation [38, 39]. Since TPEN inhibits the Fc ϵ RI-mediated translocation of PKC to the plasma membrane [31], PKC may be one of TPEN's targets affecting cytokine production. In fact, PKC contains a Zn-binding motif, and Zn is essential in maintaining PKC's structure [40]. Furthermore, its Zn-binding motif domain is required for PKCs translocation to the plasma membrane after Fc ϵ RI stimulation [41].

ZnT5 is highly expressed in mast cells, and Fc ϵ RI stimulation enhances its transcription level. *ZnT5*-KO mice have defects in mast cell-mediated delayed-type allergic reactions such as contact hypersensitivity, but not in immediate-type reactions such as anaphylaxis [42]. Consistent with these in vivo findings, *ZnT5* is required for Fc ϵ RI-mediated cytokine production, but not for mast cell degranulation.

ZnT5-KO mast cells have reduced levels of Fc ϵ RI-induced IL-6 and TNF α mRNA, and *ZnT5* is required for PKC's Fc ϵ RI-induced translocation to the plasma membrane and NF- κ B's nuclear translocation [42]. Thus, *ZnT5* is selectively required for mast cell-mediated delayed-type allergic responses, and is a novel player in PKC/NF- κ B signaling (figure 1). Furthermore, in experiments using *ZnT5*-KO DT40 cells, Suzuki and colleagues showed that *ZnT5* expressed on the ER-Golgi membrane is required for the enzymatic activity of Zn-dependent alkaline phosphatases (ALPs), which are processed from apoALPs to holoALPs in the ER-Golgi [43, 44]. Thus, *ZnT5* may act to supply Zn to the Zn finger-like domains in PKC and ALP.

These findings show that Zn and its transporters are involved in regulating degranulation and cytokine production in mast cell-mediated allergic responses, and that Zn transporters modulate the PKC/NF- κ B signaling pathway, which in turn regulates cytokine and chemokine gene expression levels.

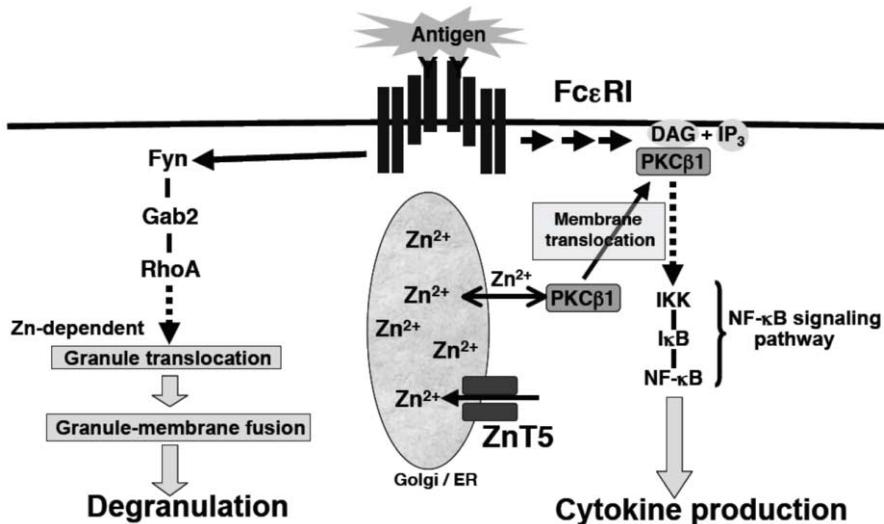


Figure 1. Zn and Zn transporters are involved in Fc ϵ RI-mediated mast cell activation. Zn is required in multiple steps of Fc ϵ RI-induced mast cell activation, including degranulation and cytokine production. Zn levels depend on Fc ϵ RI-induced granule translocation, regulated by a Fyn/Gab2/RhoA-mediated signaling pathway. Zn and ZnT5 are also required for PKC's translocation to the plasma membrane and NF- κ B's subsequent nuclear translocation, leading to the production of cytokines such as IL-6 and TNF α .

2. Zinc's Role in T Cell-Mediated Immunity

The impact of single-element nutritional deficiency on immune function has been determined by Zn homeostasis experiments in laboratory mouse and rat models at both the cellular and molecular level [45, 46], because Zn deficiency is a frequent dietary problem in humans and accompanies many chronic diseases, leading to abnormalities in the status of many other metals. It is important to note that chronic diseases such as gastrointestinal disorders, chronic diarrhea, renal disease, sickle cell anemia, cirrhosis, some cancers, cystic fibrosis, pancreatic insufficiency, and autoimmune arthritis in humans can lead to a suboptimal Zn status [47-54]. These diseases are associated with an increase in prolonged infections, which is a clear indication of compromised immunity (see chapter 11). This suggests that Zn is both directly and indirectly involved in immune cell homeostasis, activation, and development *in vivo* [49, 52] (see chapter 10). Indeed, many experimental animal models of Zn deficiency have confirmed that Zn deficiency induces thymic atrophy, lymphopenia, and compromised cell- and antibody-mediated responses, resulting in a state of immune deficiency characterized by longer, more frequent infections. This section considers Zn and immunity, mainly from the perspective of adaptive immunity—particularly the role of Zn in CD4 $+$ T cell functions important for autoimmune processes.

Zn deficiency may induce thymic atrophy by several routes, since Zn seems to regulate many molecules *in vivo*. One critical route is through glucocorticoids, particularly corticosterone, which is chronically elevated in Zn-deficient mice; adrenalectomy or removing these steroids prevents the thymus from atrophying during Zn deficiency [55, 56]. In addition, King et al. showed that a Zn-deficient diet causes a

substantial loss of CD4+CD8+ thymocytes, the thymic population known to be most sensitive to glucocorticoid-induced apoptosis [57]. Interestingly, Zn deficiency in an experimental mouse model causes an imbalance between Th1 and Th2 functions in the periphery. The production of interferon-gamma (IFN- γ) and IL-2, which are Th1 products, decreases, whereas the production of IL-4, IL-6, and IL-10, which are Th2 products, is not affected by the deficiency [58].

Zn deficiency may also induce immune deficiency, including peripheral lymphopenia, and compromise immune responses by altering gene expression in T cells and other immune cells. Indeed, a modest Zn-deficiency in mice is sufficient to induce changes in the levels of 1200 genes in T cells, by microarray analysis [59]. It is also possible that gene expression of other cell populations including dendritic cells, which present antigens to T cells, might be changed by Zn-deficiency. The altered gene expression might affect the survival and response of T cells and dendritic cells, and thereby strongly influence T cell homeostasis in vivo. In fact, mitogenic stimulation of Th0 and Th1 cells cultured in low-Zn media reduces IL-2 and IFN- γ message expression[60]. Gene expression patterns in other immune cells has been investigated in Zn-deficiency conditions. HL-60 cells, a myeloid-like human precursor cell line, survived in low-Zn culture conditions and showed upregulated TNF α , IL-1 β , and IL-8 mRNA expression upon mitogenic stimulations [60]. A potential mechanism for the effect of Zn deficiency on gene expression is tyrosine kinase signaling: Decreased intracellular Zn increases tyrosine phosphatase activity, which regulates the major tyrosine kinase signaling pathways, which have many functions in the cell, including the regulation of gene expression. Thus, intracellular Zn homeostasis is important in the response of immune cells, including CD4+ T cells.

In mouse dendritic cells exposed to the bacterial endotoxin lipopolysaccharide (LPS), which stimulates Toll-like receptor (TLR), the concentration of intracellular free Zn is downregulated, the cells mature more quickly, and they show more activation than untreated controls [61]. Artificially depleting intracellular Zn with a Zn chelator also triggers dendritic cell activation and maturation. On the other hand, artificially elevating intracellular Zn levels suppresses dendritic cells' ability to activate and mature in response to LPS. Furthermore, Zn is required for the endocytosis of Major histocompatibility complex II (MHC II) molecules expressed on the plasma membrane, and Zn inhibits MHC II vesicle trafficking from the perinuclear region to the plasma membrane [61], indicating that Zn inhibits MHC II surface expression at multiple steps.

LPS modulates the expression of a number of Slc39/Zip and Slc30/ZnT transporter molecules, which results in increased Zn transport out of the cells and reduced intracellular free Zn. For example, the Zn transporter Slc39a6/Zip6 is downregulated by LPS stimulation. This transporter, when overexpressed, suppresses dendritic cell activation and maturation, thereby inhibiting CD4+ T cell-stimulatory activity. A similar effect is observed in live animals: LPS injection reduces dendritic cells' intracellular free Zn and Zip6 expression levels, and treatment with Zn-depleting agents decreases dendritic cell activation and maturation [61]. Thus, Zn acts as an intracellular signaling molecule. LPS and other extracellular stimuli capable of changing Zn transporter expression can exert their biological activities by changing Zn levels within the cell. We call this a "late" Zn signaling event [5]. When they occur in dendritic cells, such events affect the activation state of CD4+T cells.

In immune cells, many types of molecules require Zn or are regulated by it. Numerous transcription factors and signaling molecules and more than 300 enzymes have Zn-binding motifs, such as classical Zn fingers (ZnFs), RING-domains, LIM-

domains, and PHD-domains [62]. The Src-family tyrosine kinase, Lymphocyte-specific protein tyrosine kinase p56 (Lck) is unique in that it works with a second molecule—in T cells, a CD4 or CD8 co-receptor—to create a Zn-binding motif. Although Lck function in dendritic cells has not been much described, Zn-mediated Lck binding to other functional molecules may play a role in dendritic cell activity. Lck transduces the signals required for normal T lymphocyte development and for the antigen-dependent activation of mature T cells. Lck associates non-covalently via its N-terminal region with the cytoplasmic tails of the T cell co-receptor CD4 or CD8, through direct high-affinity binding among the conserved cysteine motifs within the co-receptor tails (a CxCP motif in CD4 and CD8) and the Lck unique domain (a CxxC motif) [63, 64]. These four conserved cysteine residues coordinate a Zn-binding site that is critical for these molecules to form a complex [65-67]. A recent report showed that two Zn ions are required for the homodimerization of Lck molecules via their SH3 domains [68]. Thus, Zn modulation of Lck-binding will likely prove to be important in dendritic cells as well as in T cells.

Zn-supplying components, such as polaprezinc, have been reported to suppress autoimmune disease in animal models [69, 70]. While these results suggest that Zn can suppress autoimmune disease by inhibiting T cell activation, the details of the mechanisms involved have not been clear. We recently performed a mechanistic analysis to investigate this issue. Because stimulation by cytokines or of the TLR, which are involved in the development of autoimmune diseases, affects Zn transporter expression profiles [61, 71, 72], we first hypothesized that Zn signaling might target proteins involved in inflammation and autoimmune diseases. One such protein is STAT3, which is a signaling molecule for the pro-inflammatory cytokine IL-6. We were particularly interested in how Zn affects Th17 cells, because Th17 cell development is controlled by IL-6-induced STAT3 activation [73-77]. STAT3 also plays a role in T cell survival and in other biological reactions, and its activation state is regulated sophisticatedly [78, 79].

CD4+ T cells have been classified into Th1 and Th2 cells, which has provided a framework for understanding the roles of various CD4+ T cell subtypes in the development of autoimmune diseases [80-82]. However, recent studies have identified Th17 cells as a previously unknown effector of the CD4+ T cell response. These cells secrete several pro-inflammatory cytokines, including IL-17A [74, 77, 83-85]. In mice, a deficiency of this cytokine results in resistance to such autoimmune diseases as collagen-induced arthritis (CIA), experimental autoimmune encephalomyelitis (EAE), and the arthritis disorders that develop in F759, SKG, and IL-1 receptor antagonist-deficient mice [74, 86-89]. Until recently, the relationship between Zn and Th17 development had not been investigated.

To study this, we used induced CIA and EAE mouse disease models, since both of these autoimmune diseases are mediated by antigen-specific Th17 cells, and these cells' development is controlled by STAT3 activation. The development of both CIA and EAE was significantly suppressed by adding Zn to the animals' drinking water. In one experiment, pathogenic Th17 cells were transferred into the mice, and the mice were given Zn-supplemented drinking water. EAE development was similar in Zn-treated and control hosts, indicating that the Zn supplementation did not alter the Th17 cell-mediated immune responses, including the induced inflammation and Th17 cell activation. Thus, rather than affecting immune responses after pathogenic T cells develop, Zn inhibits the development of pathogenic Th17 cells from naïve CD4+ T cells, a process that depends on the IL-6-STAT3 signaling pathway.

Furthermore, Zn treatment decreases the serum concentrations of IL-17A, a pro-inflammatory cytokine produced by Th17 cells. However, the concentration of IFN- γ , which is primarily expressed by another activated CD4+ T cell population, Th1 cells, was similar in sera collected from Zn-treated and control mice. As would be expected from these findings, Zn supplementation decreased the number of Th17 cells in regional lymph nodes, whereas regulatory T (Treg) and Th1 cells were not markedly affected. Importantly, Zn treatment inhibited STAT3 activation in CD4+ T cells after *in vivo* treatment with IL-6. Thus, Zn supplementation suppressed Th17 cell development from naïve CD4+ T cells *in vivo*. It is possible that Zn suppresses the development of autoimmune disease by inhibiting Th17 cell development via the IL-6–STAT3 signaling axis in naïve CD4+ T cells. Importantly, we demonstrated that Zn directly binds STAT3 and inhibits its phosphorylation by JAK kinases, and does so without affecting the kinase activity of JAK proteins. Furthermore, the structure of STAT3 itself is altered by the Zn binding. Finally, we showed that the Zn binding disrupted the α -helical structure of STAT3 protein, indicating that Zn directly binds to STAT3, causing it to unfold (fig. 2).

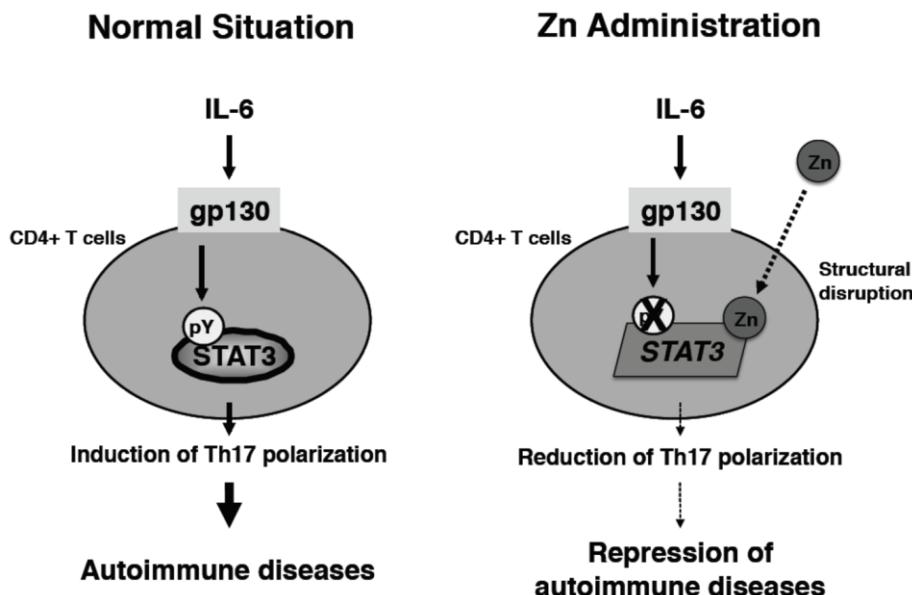


Figure 2. Zn suppresses autoimmune diseases by inhibiting STAT3 activation. Zn directly binds STAT3, altering its structure. The structurally-altered STAT3 molecule cannot effectively transduce the IL-6 signaling pathway; this pathway is critical for autoimmune diseases involving Th17 cells.

Thus, Zn directly binds and suppresses STAT3 activation, which in turn inhibits Th17 cell development, which is a critical step in the development of autoimmune diseases. We propose that Zn may provide new treatments for cancers, inflammatory diseases, autoimmune diseases such as multiple myeloma, IBD, and RA, and other conditions in which STAT3 activation affects the course of the disease. In fact, patients with such chronic diseases often show lower-than-normal serum levels of Zn [90], consistent with the idea that Zn may regulate STAT3 molecules in the pathogenesis of these diseases.

3. The Role of Zinc Homeostasis in Hard and Connective Tissues

Zn deficiency commonly causes systemic growth abnormalities, along with systemic growth retardation, dental decay, and reduced bone mass in both humans and animals [91, 92]. The concentration of Zn is relatively high in hard and connective tissues [93], suggesting its importance to their development and maintenance, although Zn's precise roles have not yet been clarified. In this final section, we discuss Zn homeostasis and its intracellular distribution in human health, in particular focusing its impact on mammalian hard and connective tissue development.

Direct evidence of the Zn transporter's involvement in developing hard and connective tissues was established by investigating *Slc39a13/Zip13* and its knockout mice [94]. *Zip13* is located in the Golgi and regulates intracellular Zn distribution. *Zip13*-KO mice display abnormalities in most mesenchyme-derived connective tissues (figure 3 and Table 1). They suffer from progressive growth retardation after 2–3 weeks of age, and have reduced bone mass due to impaired osteoblast function. They have shortened long bones, and show irregular chondrocyte column formation in the cartilage, suggesting that *Zip13* exerts a profound effect on chondrocyte differentiation.

The *Zip13*-KO mice also show abnormal tooth development in both molar and incisor teeth (Table 1). In *Zip13*-KO mice, progressive abnormalities in incisors such as deformity, malocclusions, and breakage were observed. In those mice, the crown of molar tooth develops normally, but root dentin formation is impaired (Table 1). Molar tooth development is initiated by embryonic epithelial-mesenchymal interactions, and the crown formation is completed first by postnatal ca. 10 days in mouse where epithelium-derived ameloblasts are crucially involved. Root formation is initiated after completion of crown formation, in which mesenchyme-derived odontoblasts play important roles [95, 96]. These dental phenotypes suggest that *Zip13* is not essential for crown formation but is for root formation, indicating that *Zip13* controls the functions of mesenchyme-derived cells in postnatal tooth development [97].

The *Zip13*-KO mice have fragile skin with significantly reduced dermal collagen fibrils. The physical strength of the ocular bulb is also decreased, and less corneal stromal collagen is present in the eye. Fibroblasts, which are responsible for skin and corneal development and repair[98-100], show functional dysregulation in *Zip13*-KO mice. *Zip13* controls the nuclear translocation of the Smad protein, which is phosphorylated downstream of a BMP or TGF- β receptor complex before entering the nucleus [101]. Thus, *Zip13* is involved in BMP/TGF- β signaling pathways (figure 3). Although it is unclear how *Zip13* regulates the nuclear localization of Smad proteins, it is likely that *Zip13* simply changes the Zn concentration in one of the cell compartments, or that chaperone proteins mediate the transfer of Zn from a Zn transporter to a Zn-binding molecule [102].

The *Zip13*-KO phenotypes are reminiscent of human Ehlers-Danlos syndrome (EDS) and Osteogenesis Imperfecta (OI) phenotypes [103-108]. EDS is a group of genetic disorders that affect connective tissues, and the spectrum of the disease phenotypes ranges from very mild to severe [104]. We found the siblings whose symptoms were very similar to those of *Zip13*-KO mice (Table 1), who had homologous point mutation in ZIP13's second transmembrane domain in which an aspartic acid substituted for glycine 74. When homologous, this point mutation (G74D) causes a loss of ZIP13 function. Notably, these EDS patients and *Zip13*-KO mice show phenotypes resembling Zn-deficiency [91, 92, 109]. The data obtained from *Zip13*-KO

mouse studies and EDS patients establish that the appropriate intracellular Zn distribution is crucial for the cellular functions that develop hard and connective tissues.

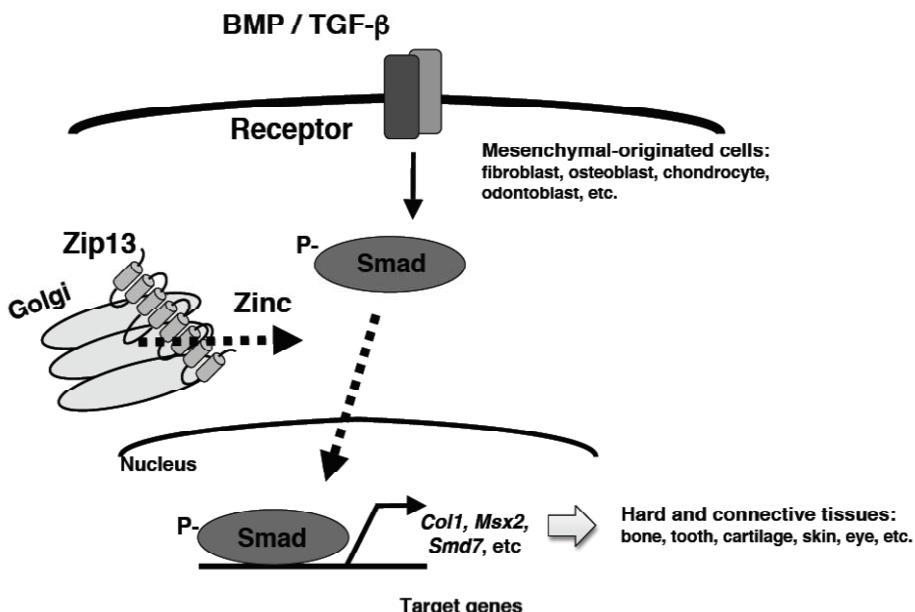


Figure 3. Zip13 is involved in BMP/TGF- β signaling pathways. Zip13 controls hard and connective tissue development in mice and humans by regulating BMP/TGF- β signaling. Zip13 is localized to the Golgi, and transports Zn from the Golgi to the cytoplasm. Zip13 affects BMP/TGF- β signaling by controlling Smad nuclear localization.

The importance of properly regulated Zn distribution for skeletogenesis and systemic growth is also demonstrated in *ZnT5*-KO mice. The *ZnT5* protein is located in the Golgi [43]. In *ZnT5*-KO mice, impaired osteoblast maturation causes decreased bone density [110]. Although *ZnT5*-KO and *Zip13*-KO mice share some phenotypic features including dwarfism and reduction of bone mass, *ZnT5*-KO mouse cartilage histology is quite different from that of *Zip13*-KO mice, indicating that, nevertheless *ZnT5*'s precise roles in skeletogenesis remain elusive, the roles and mechanisms of *ZnT5* and *Zip13* are distinct. Clearly, Zn transporter regulation of late Zn signaling, along with intracellular Zn distribution, is critical for proper cell function in hard and connective tissue-forming cells.

Interestingly, the SLC39A13/ZIP13 genetic locus was recently implicated in fasting glucose homeostasis and in type 2 diabetes risk [111]. *Zip13* levels are upregulated in Zn-deficient rat kidney and lung tissues [112]. Recently, Hojo et al reported the other Zn transporter Slc39a14/Zip14 play a role to regulate systemic growth via controlling G-protein coupled receptor (GPCR) signaling pathways, by inhibiting phosphodiesterase (PDE) activity to facilitate GPCR signaling [113]. Therefore, in addition to clarifying Zn's role in systemic growth and hard and connective tissue formation, further exploration of Zn transporter roles in late Zn signaling will likely shed light on presently unidentified disorders.

Table 1. Comparison of phenotypic and clinical features of *Zip13*-KO mice and EDS patients [94]

Category	Phenotypes in <i>Zip13</i> -KO mice	Clinical features in EDS patients
Growth and body size	Delayed growth, dwarfed body size	Delayed growth, short stature
Abnormalities in skeletal and orthopedics features	Kyphosis, decreased bone volume and osteoblast activity, shortened long bones, abnormal cartilage development	Vertebral flattening with sclerosis of the vertebral endplates, moderate osteopenia
Abnormalities in dentistry	Abnormal incisor tooth development, reduced root dentin of molar teeth, reduced bone volume of the alveolus and mandible	Hypodontia of one or a few teeth in permanent dentition
Abnormalities in craniofacial features	Enophthalmos-like appearance, down-slanting palpebral fissures	Antimongoloid eye slant with a lack of periorbital tissue
Abnormalities in dermatological features	Decreased dermis and dermal collagen; increase in skin fragility; lipoatrophy	Thin, fragile and finely wrinkled skin
Abnormalities in ophthalmological features	Fragile eye bulbs; decrease in substantia propria of cornea	Bluish or greyish sclerae; astigmatism

4. Conclusion and Perspectives

We have briefly presented recent data showing that Zn contributes to allergy and autoimmune disorders, that Zn chelator effectively inhibits anaphylaxis, and that Zn supplements suppress CIA and EAE inflammatory models. These findings may lead to future therapeutic applications for suppressing inflammatory or allergic responses. Using strategic gene targeting, we have demonstrated Zn transporter roles in various signaling pathways. For example, ZnT5 regulates PKC translocation to the plasma membrane, Zip13 controls Smad nuclear localization, and Zip14 participates in GPCR signaling by controlling PDE activity. Zn therefore functions as an intracellular signaling molecule; its intracellular status is affected by extracellular stimuli regulating changes in Zn transporter expression. Much about Zn signaling mechanisms remains to be clarified, including the method by which Zn transporters transfer Zn to individual target proteins such as PKC, Smad and PDE. We can be certain that further research will advance our understanding of Zn signaling and the intracellular transport of Zn in the immune system, skeletogenesis, and connective tissue development.

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14. Zinc and Cancer

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Abstract. Zinc is essential for cells and intracellular zinc levels are maintained within appropriate boundaries by zinc transporters. However, increasingly, changes in intracellular zinc have been observed in a number of different cancers. Furthermore, these changes in zinc levels are often accompanied by parallel alterations in the expression of different zinc transporters. In this chapter we will detail what is known about zinc and zinc transporter levels in different cancers with particular emphasis on breast cancer, which has been examined most thoroughly. It is hoped that what has been discovered regarding the involvement of zinc and zinc transporters in breast cancer will be applicable to future investigations of other cancers.

Keywords. Breast cancer, SLC39A7, SLC39A6, zinc transport, ZIP7, LIV-1

Introduction

The essential nature of zinc to cells has been described in earlier chapters. Although zinc deficiency can be detrimental, causing stunted growth and serious metabolic disorders [1], excess zinc can be equally problematic, causing toxicity to cells [2]. It is therefore important that cellular levels of zinc are well controlled. This is primarily achieved by two families of zinc transporters which transport zinc across membranes in opposing directions. The ZnT family of zinc efflux transporters (termed SLC30A) [3] transport zinc out of the cytosol and the ZIP family of zinc influx transporters (termed SLC39A) transport zinc into the cytosol [4; 5]. A further detailed description of these transporters, including a schematic figure, has been provided in Chapter 8.

The full magnitude of the role of zinc in cells is only now emerging and is altogether suggestive of an importance on a similar scale to that of calcium. The action of zinc on cells has always been considered in terms of transcriptional and DNA binding effects via zinc fingers and zinc stabilizing molecules. These effects are relatively slow in manifestation, often requiring many hours for transcription to occur. However, there is now emerging new evidence for a major role of zinc in signalling pathways and on a considerably shorter and more appropriate time scale of minutes. Although much cellular zinc is protein-associated, there is a small pool of labile intracellular zinc that is available to alter cell signalling [6].

Extracellular stimuli has been demonstrated to raise cytosolic zinc in minutes by the release of zinc from the endoplasmic reticulum (ER) store, altering cell signalling

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and classifying zinc as a second messenger [7]. This zinc release from stores, termed a ‘zinc wave’ [8], was described as the means by which zinc causes widespread phosphatase inhibition [9] and explained how cellular zinc can have such truly extensive effects. Protein kinases regulate a myriad of signalling pathways and likewise, phosphatases, by inactivation of kinases, can have equally broad cellular effects. This widespread impact of zinc on cellular processes has been reinforced by demonstrations of increased phosphorylation of many tyrosine and MAP kinases [3, 9] caused by inhibition of phosphatases rather than stimulation of kinases [10] and produced as a result of cytoplasmic zinc release from stores (see chapter 6).

1. The LIV-1 Family of ZIP Transporters

The SLC39A family is divided into 4 separate groups, as demonstrated by the dendrogram (Figure 4) in Chapter 8. There are 9 human members of the SLC39A family which belong to the LIV-1 subfamily [5]. Increasingly it seems to be the aberrant expression of this family of zinc transporters that is primarily implicated in the increasing number of disease states that have zinc transporter involvement. All ZIP transporters are predicted to consist of 8 transmembrane (TM) domains with conserved histidine residues within TM IV and V that are believed to be involved with zinc transport [5]. Importantly, the 9 human members of the LIV-1 subfamily contain all these motifs as well as additional histidine rich regions on the N-terminus and extracellular loop between TM II and III [5] with a considerable increase in the number of histidine residues, although the exact role of these histidine residues has not yet been determined. The LIV-1 family is grouped into a separate subfamily [5] due to the presence of an additional motif in TM V, which is highly conserved (Figure 1).

ZIP7	SLC39A7	KE4	358	LHEV PHEV GDF AILV
ZIP10	SLC39A10		713	CHELPHEL GDF AVLL
ZIP6	SLC39A6	LIV-1	629	CHELPHEL GDF AVLL
ZIP14	SLC39A14		376	OEEFPHEL GDF VILL
ZIP4	SLC39A4		536	CHELPHEL GDF AILL
ZIP8	SLC39A8	BIGM103	343	OEEFPHEL GDF VILL
ZIP5	SLC39A5		423	CHELPHEL GDF AMLL
ZIP12	SLC39A12		543	CHEIPHEM GDF AVLL
ZIP13	SLC39A13		257	LHEIPHEV GDF AILL

Figure 1. LIV-1 family of ZIP transporters nomenclature and conserved motif in transmembrane domain 4. Black shading indicates identity and grey shading complementary residues.

Most of the LIV-1 family proteins are present on the plasma membrane (Figure 2) and transport zinc into cells from the extracellular space [10; 11; 12; 13], whereas ZIP7 is located on the endoplasmic reticulum membrane (Figure 2) and transports zinc into the cytosol from intracellular stores [14], agreeing with the location of the *Arabidopsis* homologue of ZIP7, IAR1 [15]. ZIP7 has also been demonstrated to reside

on the golgi membrane [16] as has ZIP13 [17], another LIV-1 family member that has been shown to reside on internal membranes.

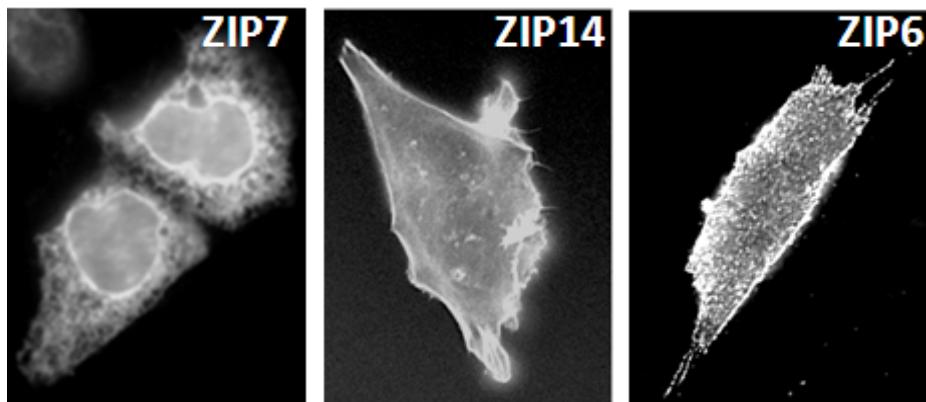


Figure 2. Cellular location of different ZIP transporters, amended from [18]. ZIP6 and ZIP14 are present on the plasma membrane of non-permeabilised cells whereas ZIP7 is localised internally on the endoplasmic reticulum membrane in permeabilised cells.

2. Zinc Handling in Cells by Zinc Transporters

There is a relatively large number of ZIP and ZnT transporters present in cells [3] [4] and yet the exact way that they interact to control the level of intracellular zinc is still not fully understood. However, three pieces of independent data has recently helped shed light on the mechanism of zinc transport within the cell.

Firstly, the presence of a zinc wave was demonstrated in mast cells as a direct result of cross-linking of the high affinity immunoglobulin E receptor ($\text{Fc}\xi\text{R}$) which induced release of free zinc from the perinuclear/endoplasmic reticulum area of cells [7]. This zinc wave manifested itself as a release of zinc from an intracellular store, such as the endoplasmic reticulum, in the form of a wave throughout the cytoplasm, although the exact mechanism of its release from this compartment was not determined in this study. This zinc wave was dependent on both calcium influx and activation of MAP kinase and defined zinc as a second messenger involved in intracellular signalling events directly resulting from an extracellular stimulus. Importantly, this zinc wave was demonstrated responsible for the previously described ability of zinc to inhibit the action of a widespread number of phosphatases [19], which has considerable impact on the activation of molecules such as tyrosine kinase receptors that rely on dephosphorylation by phosphatases for their inactivation and removal.

Secondly, a model for zinc trafficking within cells was proposed using computational modelling of experimental data obtained from cultured cortical neurones challenged with added zinc in the medium [20]. Of the three models that they tested for ability to match intracellular free zinc transients and total zinc content, only one model was able to reproduce the experimental results with accuracy. This successful model predicted that intracellular zinc was associated in the cytoplasm with a "muffler" or buffer that had a high affinity for zinc, perhaps molecules such as metallothionein and glutathione, that would allow zinc to be strongly buffered (see chapter 4). Subsequently, the buffer-associated zinc would then be distributed into a deep cellular store, such as

the endoplasmic reticulum compartment, before release to the cytoplasm. This predicted model is consistent with the zinc wave proposed above, provided the subsequent release of zinc from intracellular stores was in the form of a wave throughout the cytoplasm and originated from the endoplasmic reticulum.

Thirdly, our group has begun to define a previously unidentified role for zinc in contributing to oestrogen receptor dependent and independent forms of endocrine resistant breast cancer through its capacity to sustain the activity of growth factor signalling. Zinc is known to cause inactivation of several phosphatases, many of which are involved in the dephosphorylation and inactivation of Epidermal Growth Factor Receptor 1 (EGFR), receptor tyrosine-protein kinase ErbB2, Insulin-like Growth Factor Receptor 1 (IGF1-R) and c-Src gene from the rous sarcoma virus (Src) [9], all of which are signalling molecules of known importance in the development of tamoxifen-resistant breast cancer [21]. Zinc can also inhibit plasma membrane Ca^{2+} -ATPase (PMCA), a calmodulin-regulated P-type ATPase that has a key role in the control of intracellular Ca^{2+} , at physiological concentrations ($\text{IC}_{10} = 4\text{pM}$; [21]) leading to a rise in $[\text{Ca}^{2+}]_i$, which has been associated with migration of breast cancer tumour cells [22]. We have demonstrated that our model of tamoxifen-resistant (TamR) breast cancer cells, described in more detail in section 4.4, have increased concentrations of loosely ligated intracellular zinc compared to their hormone responsive (wild-type) counterparts and an accompanying increased expression of zinc transporter ZIP7 [23]. Consequently, treatment of these cells with physiologically relevant levels of zinc promotes the Src-dependent stimulation of tyrosine kinase receptor signalling pathways, contributing to their aggressive phenotype by promoting tumour cell growth and motility [23].

We also demonstrated that this activation of signaling pathways was entirely due to ZIP7 dependant release of zinc from the endoplasmic reticulum using fluorescent zinc dyes such as Zinquin, Fluozin-3 and Newport Green. In the presence of ZIP7, treatment with zinc caused activation of numerous signaling pathways such as EGFR, ErbB2, IGF1-R, Src, MapKinase and serine/threonine protein kinase AKT, which was paralleled by increases in cell growth rate and invasion. These effects were demonstrated to be entirely due to the ZIP7-dependent release of zinc from the endoplasmic reticulum, fitting with the 'zinc wave' mentioned above, as they were abolished when ZIP7 was removed using siRNA technology [23]. This data provided good evidence that ZIP7 was essential for the zinc wave and the subsequent zinc-induced inhibition of phosphatases.

Taken together these three separate pieces of data suggest a mechanism for zinc distribution in cells [18] that is represented in Figure 3. Essentially, extracellular zinc entering the cell, probably utilizing a ZIP transporter, is immediately buffered within the yet undefined 'zinc muffler' that will consist of agents such as metallothionein and glutathione, well known for their zinc buffering ability. Subsequently the zinc will be sequestered into the intracellular zinc store, such as the endoplasmic reticulum, probably utilizing a ZnT transporter. Once the zinc has arrived in the internal store it will be available for release into the cytoplasm by a ZIP7-mediated zinc wave [23] resulting in the widespread inhibition of phosphatases that has been previously attributed to zinc [9]. This mechanism explaining how zinc is handled within cells has considerable implications for treatment of diseases such as cancer that often exhibit increased intracellular zinc and therefore aberrant activation of many different tyrosine kinases. This now provides the relevant ZIP and ZnT transporters as new and novel targets for inhibiting tyrosine kinase activation in diseases such as cancer.

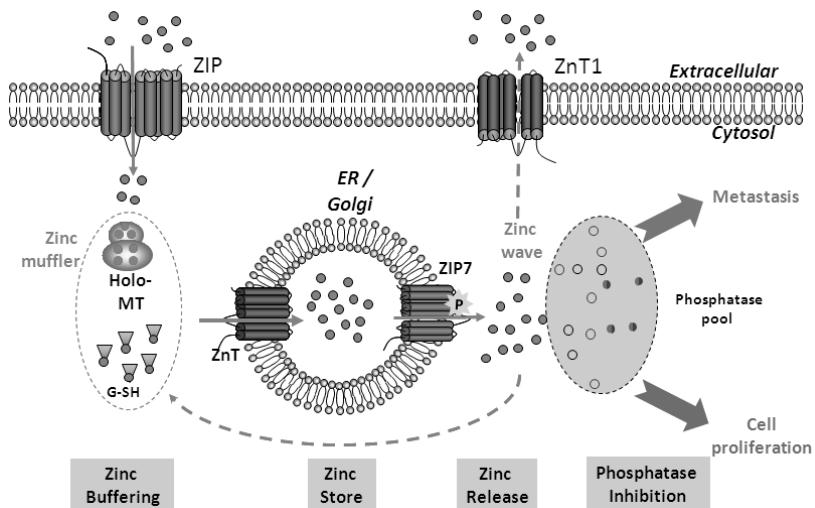


Figure 3. Schematic showing zinc handling in cells by zinc transporter ZIP7. Zinc entering cells, possibly by a ZIP transporter, is buffered in the zinc muffler before being sequestered into the ER store, possibly by a ZnT transporter. Zinc is released from the store by ZIP7 in the form of a zinc wave which causes inhibition of phosphatases and the activation of downstream signalling pathways leading to proliferation and metastasis. Excess intracellular zinc can be excreted from the cell by ZnT1 or reabsorbed by the zinc muffler.

This mechanism may also help to explain why there are an apparent large number of the LIV-1 family zinc transporters. The plasma membrane located ZIP transporters are often expressed in a tissue specific manner whereas ZIP7, located on intracellular stores, is ubiquitously expressed in cells [14]. If this mechanism holds true for zinc handling in all cells then it may help to explain the hierarchy of certain ZIP transporters and why, for example, increased expression of ZIP7 is consistently associated with such bad outcomes in clinical cancer databases, as detailed in section 4.2.

3. Zinc Status in Cancer

Over the years many groups have tested serum zinc and plasma as a potential biomarker for different cancers. However, the results obtained have been extremely conflicting and have led to a considerable amount of confusion. Some studies have documented increases of zinc as an indicator of unfavorable outcome whereas others have documented decreases of zinc associating with poorer outlook. For example, serum or plasma zinc decreases have been observed in head and neck cancer [24], cervical cancer [25], lung cancer patients [26] and gynecological cancers [27]. However, there are also many epidemiological studies looking at zinc levels in patients with breast, lung, stomach and prostate cancer that have been documented which also found conflicting results [28]. Additionally, zinc levels in hair samples have been examined and have not produced such varied results but this may be due to the small sample number. These studies demonstrated decreased zinc in scalp hair from patients with ovarian cancer [29] or lung cancer [30].

However, in an effort to produce more robust indications of zinc status in cancer samples, some groups have extended their observations to examining levels of zinc in actual cancer tissue samples. This has revealed a decreased zinc level in kidney carcinoma and unaltered zinc in a variety of other different cancer tissues [31]. Due to

some observed decreases of serum zinc in cancer, zinc supplementation of cancer patients has also been considered. Zinc has already been suggested to show an inverse relationship with cancer development in human cutaneous fibroblasts by significantly decreasing DNA strand breaks after exposure to UVA1 radiation [32]. Data also suggests that zinc supplementation should have beneficial effects on cancer by decreasing angiogenesis and induction of inflammatory cytokines while increasing apoptosis in cancer cells [33]. However, although a higher intake of zinc has been shown to have an inverse relationship with breast cancer [34], most observations gave confusing results. It was noteworthy that the most marked results were obtained when the control subjects were actually zinc deficient rather than on an adequate zinc diet. One explanation for this variety of effects is that most of the studies to date have used zinc supplementation in combination with other nutrients which makes it difficult to interpret the results. Since zinc supplementation is associated with decreased oxidative stress and improved immune function that alone may account for any observed cancer preventive activity of zinc [35].

3.1. Zinc Status in Breast Cancer

Many studies have also examined the relationship between zinc in serum or plasma with breast cancer. Decreased serum zinc has been observed in breast cancer patients in a number of studies [29 ; 36; 37 ; 38] whereas serum zinc increases have also been seen in breast cancer patients [39] while others showed no changes [30]. Zinc levels have also been examined from scalp hair as above but from breast cancer patients and demonstrated either decreased zinc [29] or no difference in hair zinc [30].

Interestingly, the confusion observed with varying levels of zinc in different cancers is much less when zinc levels in the actual breast cancer tumours have been measured rather than the testing of serum or plasma. For example, when testing the actual zinc measurement in breast cancer tissue, all results seem to be consistent with elevated levels in cancer tissue compared to normal breast tissue [31; 40] with increases often double those of benign breast tissue [41]. These results do seem to indicate that the increase of zinc in breast cancer tissue is quite robust. This is reinforced by a widely used model of breast cancer in the rat, induced by N-methyl-N-Nitrosourea (MNU), where the zinc concentration in mammary tumours was observed to be as much as 19 fold increased compared to normal mammary tissue and irrespective of the zinc intake [42]. This data has been supported by another study demonstrating increases of 12 fold zinc in mammary tumours using this MNU rat model [43]. Furthermore, increased MT expression has also been observed in breast cancer [41] which is consistent with the elevation of zinc observed in this disease state.

The lowering of serum zinc and increased tumour zinc in breast cancer may seem to be a conflicting phenomenon. However, it is well known that zinc is essential for the functioning of a number of cyclins and therefore the associated cell cycle progression through the G1 and G2 phases of the cell cycle [44] is reliant on zinc. Consequently, it is entirely possible that during the uncontrolled growth associated with cancers, the cancer cells may be using more zinc than they normally use and that this generates a need to replenish zinc from plasma. This mechanism may explain the observed decreased levels of zinc in serum and plasma observed in some cancer patients while the tumour zinc is actually considerably elevated above normal levels. This will now need further verification experimentally.

3.2. Zinc Status in Prostate Cancer

Patients with prostate cancer have reduced plasma zinc compared to normal patients whereas those with non-cancerous prostate disease have considerable elevation of plasma zinc [45]. This finding is reinforced by a number of other studies showing good evidence that the zinc level in the prostate decreases significantly (more than 50%) in prostate cancer whereas the zinc level increases dramatically in prostate disease [46; 47; 48; 49; 50; 51; 52]. This change in zinc status during cancer has been shown to be due to the malignant prostate cells losing the ability to accumulate zinc [53; 54] mainly caused by the decrease in zinc transporter ZIP1[54].

The decision of whether to supplement zinc in patients at risk of prostate cancer is however quite complex because taking zinc supplements of over 100mg per day or taking them for over ten years has been shown to double the chance of developing prostate cancer [55] rather than providing protection.

4. Role of Zinc Transporters in Cancer

It is clear from these studies above examining patient zinc levels in cancer that the situation is quite complicated, possibly varying according to the cancer type. Further detailed examination is needed to discover the mechanism of action of zinc in cancer in order that more appropriate clinical targets can be defined. One way to achieve this is to examine the expression and location of the myriad of zinc transporters that are present in the different cell types in order to gain an understanding of how they interact together to bring about an alteration in zinc status.

Zinc transporters are the means by which zinc moves across cellular membranes and therefore aberrant expression of zinc transporters will likely change the level of zinc in cells which in turn may lead to disease states such as cancer. There is paucity of examination of cohorts of ZIP zinc transporters in different cancers. Most critical examination has been performed in relation to breast cancer, which will be dealt with in more detail below, followed by a short discussion of the recent discoveries in prostate cancer and other cancers.

4.1. Zinc Transporters in Breast Cancer

The first ZIP transporter to be linked to breast cancer was ZIP6 (LIV-1/SLC39A6), a member of the LIV-1 family of zinc transporters, which was shown to be oestrogen-regulated and present in increased amounts in oestrogen-receptor positive breast cancers that spread to the lymph nodes [56; 57]. More recently, this association of ZIP6 with oestrogen receptor positive breast cancers has been substantiated by larger scale analysis of breast cancer specimens where it has been proven to be such a reliable marker of oestrogen receptor positive cancers [58; 59] that it is one of the genes used routinely to distinguish the luminal A type of clinical breast cancer [60; 61].

Additionally, we have shown a role for ZIP7 as a hub in the pathway of zinc-mediated growth factor signalling [23] through its increased expression in our different breast cancer models of anti-hormone resistance [62]. The responsibility of ZIP7 to release zinc from stores in these cells, along with the phosphatase inhibition that this causes, suggests ZIP7 could be responsible for the prolongation of growth factor signalling and likely to be of regulatory importance in a range of disease states

characterised by the increased activation of tyrosine kinases. The role of both ZIP6 and ZIP7 in breast cancer is covered in more detail below.

4.2. Zinc Transporter ZIP7 in Breast Cancer

In order to try to define the mechanism of action of ZIP7 (SLC39A7/HKE4), it was necessary to test whether the genetic manipulation of ZIP7 was consistent with a corresponding alteration in intracellular zinc levels. We have used our tamoxifen-resistant MCF-7 cells (TamR cells [63], described in section 4.4 below) to investigate the role of ZIP7 in cells as not only do they have increased expression of ZIP7 without other ZIP transporters [62] but they also have approximately double the level of intracellular zinc than the wild-type MCF-7 cells, as examined using the fluorescent zinc specific dye Newport Green [23]. We were able to investigate the intracellular zinc levels in these conditions by loading the MCF-7 breast cancer cells with zinc specific dyes and either reading the population fluorescence by FACS analysis or observing zinc concentration in single cells by fluorescent microscopy. Both techniques produced the same results, verifying that manipulation of ZIP7 did cause changes in intracellular zinc levels [23]. For example, we observed that transfecting MCF-7 cells with ZIP7 for 24 hours produced a 50% basal increase in intracellular zinc in MCF-7 cells, comparable to the basal level observed in our TamR cells. We also observed a considerably enhanced increase in intracellular zinc levels when ZIP7 transfected MCF-7 cells were stimulated with zinc which was not present in control MCF-7 cells. Furthermore, the presence of siRNA for ZIP7 reduced any zinc-induced increases in intracellular zinc as observed by Fluozin-3 or Zinquin fluorescence. The presence of siRNA for ZIP7 additionally reduced the zinc-induced increase of intracellular zinc when measured by FACS analysis using cells loaded with Newport Green.

Importantly, we also demonstrated a role for ZIP7 in the aggressive phenotype of TamR cells by removal of ZIP7 using siRNA [23]. Excitingly, in the presence of ZIP7 siRNA, the previously observed activation of EGFR, Src, IGF1-R and AKT was not seen. Furthermore, the ability of TamR cells to migrate across matrigel, a protein mixture resembling the extracellular environment, was considerably reduced in the presence of siRNA for ZIP7. Interestingly, the converse was also true. When wild-type MCF-7 cells were transfected with a construct expressing recombinant ZIP7 for 24 hours there was evidence of activation of EGFR, Src and IGF1-R which was paralleled by an increase in motility. The model above (Figure 3), explaining a mechanism for zinc release from intracellular stores by ZIP7, clarifies the key role that ZIP7 plays in the control of intracellular zinc homeostasis due primarily to its location on the endoplasmic reticulum (Figure 4). Furthermore, the proven ability of zinc released intracellularly to inhibit a wide range of phosphatases [8; 9; 19; 64] explains how the presence of abnormally high zinc levels in cells is able to keep many tyrosine kinase signalling pathways activated, which, in the case of cancer, will lead to increased growth and invasion [23] as a further consequence of activation of AKT [65] and inhibition of glycogen synthase kinase 3, GSK-3 β [66; 67].

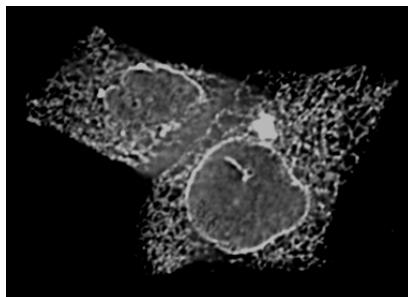


Figure 4. Over expression of recombinant ZIP7 in permeabilised MCF-7 cells demonstrating location in the endoplasmic reticulum with strong perinuclear association. The ZIP7 has a C-terminal V5 tag [14] that was probed with an anti-V5 antibody and Alexa-Fluor 488 secondary antibody.

This key role of ZIP7 is supported by its increased expression in clinical breast cancer samples where it is in the top 10% of genes over-expressed in poor prognostic breast cancer states, those with relapse or death after 5 years, those with lymph node spread and those with invasive cancer (www.Oncomine.com). Furthermore, we have correlated ZIP7 mRNA expression with proliferation-related Ki67 antigen, ErbB3, lymph node spread and signal transducer and activator of transcription 3 (STAT3) in a clinical breast cancer series [62], all agents that have been linked to poor outlook in breast cancer, supporting a role for ZIP7 in breast cancer.

Increasingly in breast cancer, aberrant growth factor signalling supports rapid tumour cell proliferation and loss of therapeutic response to anti-hormonal drugs. It is clear that multiple growth factors, their receptors and downstream signalling elements can promote tumour growth making it difficult to judge which signalling cascades to target [68] and illustrated by the disappointing clinical results obtained with the EGFR inhibitor Gefitinib despite high EGFR levels in many breast cancer specimens [69; 70]. Importantly, we have shown that zinc transporter ZIP7 facilitates growth factor signalling by EGFR, ErbB2, ErbB3, ErbB4, IGF1-R and c-Src through its ability to inhibit phosphatases [23]. This is an exciting result and increased expression of ZIP7 in breast cancer now provides a novel target for blocking multiple anti-homone resistant signalling pathways.

Significantly, the activation of receptor tyrosine kinases has been previously shown to be a result of zinc-induced inhibition of the protein tyrosine phosphatase 1B (PTP1B) [9]. Known substrates for phosphatase PTP1B include EGFR, insulin receptor substrates 1 and 2 (IRS-1 and IRS-2), IGF-1R and Src [64] and therefore inhibition of a molecule such as PTP1B in our TamR cells might have a significant effect on the activation of signalling pathways by preventing their dephosphorylation and subsequent inactivation. There is little information available in the literature concerning zinc induced effects on signaling molecules however, there is some evidence to suggest that zinc can induce gene expression as a downstream effect of activating growth factor signalling pathways, including activation of EGFR at Y845 by transactivation via c-Src in human epidermoid carcinoma A431 cells and human bronchial epithelial cells, BEAS [71; 72].

Additionally, however, we have recently shown that TamR cells harness IGF-1R/IRS-1 signaling to support EGFR activation [21] and a prolongation of such responses would thus also aid aggressive cell behavior. A role of zinc in insulin and insulin-like growth factor receptor-1 signaling has already been documented [19; 67; 73; 74] as well as an ability to induce activation of the non-receptor tyrosine kinase,

Src, which will have additional consequences leading to increased proliferation, angiogenesis, survival and increases in motility and invasive capability [71]. Furthermore, insulin-like growth factor-1 receptor and the insulin receptor are present in all breast cancer subtypes in their activated form and this is directly related to poor survival [75]. This could be further evidence of the increased intracellular zinc in breast cancer tumours.

Despite this central role of ZIP7 in cells, it is not known how ZIP7 functions to transport zinc and what switches its zinc transport capability on and off. Importantly, three recent whole genome phosphorylation screens have discovered that two adjacent serines (S275/S276) on ZIP7 are phosphorylated [76,78] and analysis of these sites, using the online software tool ELM [79], confirms the potential for phosphorylation by protein kinase CK2 with the extra acidic residues within these motifs likely to enhance CK2 phosphorylation capability. Interestingly, work by others has shown that DMAT, a CK2 inhibitor, causes tamoxifen resistant cells to die by apoptosis [80] and our unpublished data additionally shows DMAT has no such effect on wild-type MCF-7 cells. Protein kinase CK2 has broad cellular effects in cells [81] and is increased in all cancers that have been tested to date [82]. CK2 primarily promotes cell survival [83] by driving the PI3K/AKT pathway and stabilizing the phosphatase and tensin homolog, PTEN, activating AKT, increasing NF κ B transcription, increasing the activation of the cell adhesion molecule β -Catenin and activating Wnt signalling, making caspases resistant to cleavage and increasing DNA repair. Crucially, zinc has similar effects encouraging thinking that CK2 and ZIP7 are involved together in zinc transport. The potential role of CK2 in activating ZIP7 now provides a clinical opportunity due to the availability of small molecule CK2 inhibitors and their preliminary success in clinical trials [84].

4.3. Zinc transporter ZIP6 in breast cancer

ZIP6 (SLC39A6/LIV-1), the first member of the LIV-1 family to be described [10], has been known to be associated with oestrogen receptor positive breast cancer since 1993 [57] and present in increased amounts in oestrogen-receptor positive breast cancers with an ability to spread to the lymph nodes [56]. When localized in cells to the plasma membrane it has always been observed in lamellipodiae, consistent with a potential role in migration and invasion (Figure 5). More recently, ZIP6 has become a reliable marker of oestrogen receptor positive cancers used to distinguish the luminal A type of clinical breast cancer. Furthermore, in zebrafish embryos, ZIP6 was shown to be the downstream target of the transcription factor STAT3 [85], which has a proven role in the development of cancer [86] and metastasis [87]. This work also found that ZIP6 was essential for the nuclear localisation of the transcription factor Snail, a major factor in the epithelial to mesenchymal switch due to its ability to down regulate the expression of genes associated with cell adhesion, suggesting that ZIP6 could be a link between cancer and development [88] and raising the question of whether any other zinc transporters have a similar role. In fact ZIP10, another zinc transporter belonging to the LIV-1 subfamily and with the closest sequence similarity to ZIP6 [5] has recently been linked to the invasive capability of breast cancer cells [89].

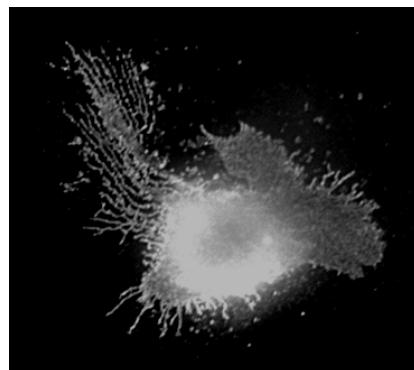


Figure 5. Overexpression of recombinant ZIP6 in non-permeabilised MCF-7 cells demonstrating location on the plasma membrane and especially the lamellipodiae. The ZIP6 had a C-terminal V5 tag [10] that was probed with a V5 antibody and Alexa-Fluor 488 secondary antibody.

A schematic explaining the potential role for ZIP6 in metastasis and EMT (epithelial to mesenchymal transition) is shown in Figure 6. Once STAT3 has been activated by growth factors or other agents, ZIP6, which is the downstream target of STAT3 [85], will be able to influx zinc into cells from its position on the plasma membrane [88]. The subsequent increases in intracellular zinc can together activate Snail [90] by inhibition of GSK3 β [67] and/or activation of Akt [74; 91] resulting in loss of E-cadherin gene expression, cell detachment and EMT. The involvement of ZIP6 with Snail has been described by the observation that siRNA for ZIP6 reduces HeLa cell invasion via a Snail pathway [48]. This mechanism may explain the previously observed increased expression of ZIP6 in breast cancers that have metastasized to lymph nodes [92].

4.4. Zinc Transporters in Anti-Oestrogen Resistant Breast Cancer

The primary clinical treatment for oestrogen receptor positive breast cancer is anti-hormones such as tamoxifen. These agents effectively stop the growth of many of these cancers, with 40% being *de novo* resistant. However, with time, resistance can also develop in initially responsive cancers which can then grow again with a more aggressive phenotype having acquired resistance. In order to better understand the mechanisms underlying the occurrence of resistance we have developed a unique panel of anti-oestrogen resistant cell lines derived from the oestrogen receptor positive human breast cancer cell line MCF-7 [63]. Our tamoxifen resistant cells (TamR) exhibit an increased rate of growth and increased ability to invade in the presence of tamoxifen by efficiently using alternative signalling pathways such as EGFR [63], Src[93] and IGF1-R [21] allowing them to exhibit this more aggressive phenotype [94]. Interestingly, our faslodex resistant cell lines (FasR) can utilize the previously unused signalling pathway driven by the proto-oncogene c-Met [95] when provided with exogenous hepatocyte growth factor ligand (HGF) to drive their invasive behavior.

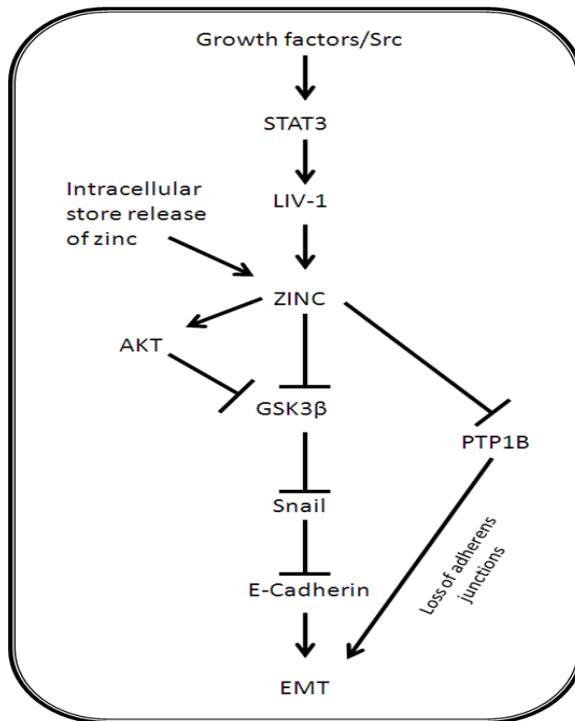


Figure 6.Schematic explaining the role of zinc and zinc transporter ZIP6 in EMT. The stimulation of ZIP6 by growth factors and STAT3 will increase intracellular zinc which in turn can inhibit GSK3 β directly and indirectly by activation of AKT. Inhibition of GSK3 β will allow Snail to remain active and in the nucleus to prevent the expression of E-cadherin and other adherence genes. The increase in zinc will also encourage EMT by inhibition of PTP1B and loss of adherens junctions.

We have used PCR to investigate the expression of all 9 human LIV-1 family members in both our tamoxifen (TamR) and fulvestrant (FasR) resistant cell lines and compared them to the wild-type MCF-7 cells (Figure 7) in an effort to shed light on any potential role that individual family members may play in the development of anti-hormone resistant breast cancer [62]. The 2 family members that were altered the most significantly were ZIP7 and ZIP8, with ZIP7 elevated in both resistant states. ZIP4 levels were undetectable and the levels of ZIP5, ZIP10, ZIP12 and ZIP13 were relatively low. The levels of ZIP6, ZIP5, ZIP12 and ZIP13 were either unchanged or reduced in the resistant cell lines. ZIP14, although present in low amounts, was modestly increased in the resistant cells and ZIP10 decreased only in TamR cells. These results indicate that a number of LIV-1 family members (ZIP4, ZIP5, ZIP6, ZIP10, ZIP12 and ZIP13) are not increased on acquisition of endocrine resistance. Both ZIP8 and ZIP7 are elevated in one or both anti-hormone resistant cell lines respectively, suggesting a possible role for these two family members in the development of resistance. In this regard we have demonstrated an important role for ZIP7 and zinc in driving growth of TamR cells [23] which has already been discussed in 4.2 above.

We have previously investigated the levels of the LIV-1 family members in a series of tumour samples [62] from patients at the time of presenting with primary breast cancer. The expression of ZIP4, ZIP12 and ZIP13, was undetectable in these samples, the amount of ZIP7, ZIP8, ZIP10 and ZIP14 showed little variation and the levels of ZIP6 and ZIP5 showed the most heterogeneity with variations from low to medium and high values. The statistical significance of these values was compared to a number of common indicators of breast cancer progression and grade [62]. ZIP6 was confirmed as an oestrogen regulated gene and a prognostic marker of subsequent endocrine response. ZIP6 also had an inverse relationship to EGFR and a positive association with two other erbB receptor tyrosine kinase members, ErbB3 and ErbB4, and another growth factor receptor, IGF1-R, also indicative of an endocrine responsive phenotype [62]. No other LIV-1 family member exhibited the same profile as ZIP6.

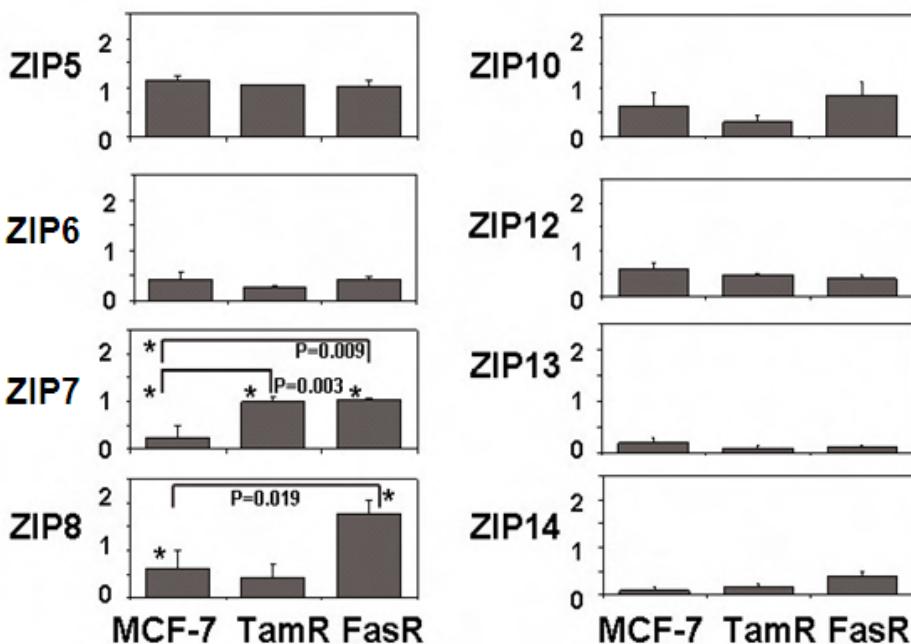


Figure 7. Gene expression of LIV-1 family members in anti-oestrogen resistant cell lines as determined by PCR, adapted from [62]. TamR=tamoxifen resistant MCF-7 cells, FasR=faslodex resistant MCF-7 cells.

ZIP5 and ZIP10 both showed a positive association with oestrogen receptor but, due to their apparent low levels of expression, may not have had any useful clinical relevance. No other family member had an association with EGFR or ErbB4, however, ZIP7 and ZIP10 both showed a positive association with ErbB3, which has recently been shown to have a role in faslodex resistant breast cancer [96]. ZIP8 behaved differently to the other family members showing a negative association with both oestrogen receptor and ErbB2. Another observed relationship was a positive association of ZIP7 with the well known proliferation marker Ki67 and those cancers with increased lymph node involvement [62] although this was not seen for any other family members. This data suggests that of all the LIV-1 family members, increased

expression of ZIP7 and ZIP8 are the most indicative of an adverse response and an increased likelihood of the development of acquired resistance.

We have recently analyzed the expression of a broader panel of zinc related genes extending studies to four of our acquired anti-hormone resistant cell lines by Affymetrix gene microarray analysis and compared expression to that of wild-type MCF-7 cells. These results are given in Table 1 where mRNA levels in acquired resistant cells are expressed as fold changes from MCF-7 cells. The cell lines we have used are all derived from MCF-7 cells, and comprised tamoxifen resistant (TamR), faslodex resistant (FasR), oestrogen deprived (X-MCF) and gefitinib (EGFR inhibitor) and tamoxifen resistant (GEF-TamR) cells. We observed a twofold increase in ZIP7 and a threefold increase in ZIP8 in TamR cells which confirmed our previous PCR data [23]. Interestingly, ZIP7 was also increased in the GEF-TamR suggesting that these cells may maintain reliance on ZIP7 when they also acquire resistance to endocrine blockade. The most striking observation was that ZnT5 (SLC30A5) was increased considerably in all acquired resistance cell lines. ZnT5 has been located on the golgi or endoplasmic reticulum membranes [97] and transports zinc into these stores. Interestingly, as part of the mechanism of how zinc is handled in cells (Figure 3) and discussed above, it is unknown how the zinc is transported from the 'muffler' into the endoplasmic reticulum. It would therefore be appealing to speculate that ZnT5 may have a role to play in this mechanism and its increased expression in these cells may be indicative of increasing the zinc availability in the intracellular zinc stores in acquired resistant cells to enhance the zinc store release by ZIP7 and possibly ZIP8.

Table 1. Fold change in gene expression compared to wild-type MCF-7 breast cancer cells

Gene	TamR	FasR	X-MCF	GEF-TamR
ZnT5	3.05	3.65	4.59	3.03
ZIP8	1.43	3.35	3.26	1.66
CK2α	1.86	4.85	1.61	1.73
MT2A	3.35	2.28	1.83	1.45
ZIP14	1.99	2.31	1.63	2.36
ZIP6	1.05	2.04	4.03	2.13
MT1F	2.52	2.08	1.62	1.02
ZIP7	2.2	1.19	1.02	2.14
MT1H	2.22	1.33	1.4	1.04
MT1E	1.69	1.32	1.44	1.31
CK2β	1.2	1.21	1.07	1.4

TamR = tamoxifen resistant MCF-7 cells, FasR = faslodex resistant MCF-7 cells, X-MCF = oestrogen deprived MCF-7 cells, GEF TamR = gefitinib resistant TamR cells, CK2 = protein kinase CK2, MT = metallothionein. Only fold changes of greater than 1.5 were considered significant.

Further evidence that zinc may be involved in these anti-hormone resistant cell lines is the observed increased expression of a number of metallothioneins which is suggestive of increased intracellular zinc driving more aggressive metastatic tumours [98]. For example, MT2A was increased three or two fold in the TamR and FasR cells respectively. These cells are quite aggressive and, interestingly, increased MT2A has also been observed in grade 3 breast cancer rather than grade 2 or 1 [99] suggesting a link to more aggressive tumour behaviour. MT1F, also increased in these cells, has been directly linked with poor histological grade [100].

It is also obvious that ZIP8 is more than threefold increased in the FasR and X-MCF cell lines. ZIP8 has been located on intracellular membranes such as lysosomes [101] and mitochondria [102]. It would be attractive to consider whether there is also a role for zinc in these cells versus the role for ZIP7 in TamR cells and speculate that the

zinc may take an alternative route to travel through these cells with reliance on ZIP8 to release it from alternative stores. This now needs to be investigated experimentally. However, the situation is still complex because two ZIP transporters located on the plasma membrane, ZIP6 and ZIP14, are also differentially increased in these cell lines, the latter also suggested by PCR in our FASR cells, which may be indicative of increased zinc uptake capability. We also examined whether there were any changes in protein kinase CK2 due to its potential to phosphorylate ZIP7. CK2 is a heterotetrameric serine/threonine kinase [103] and the α -subunit is the catalytic subunit. It is noteworthy that CK2 α (CSNK2A1) was increased in all cell lines whereas the regulatory subunit CK2 β (CSNK2B) was not, suggesting that CK2 may play a role in zinc transport.

4.5. Zinc Transporters in Breast Cancer Patient Response

In order to begin to extend these Affymetrix gene expression investigations into clinically relevant material we have interrogated the expression of these zinc related molecules in patient samples and compared the effects of their expression levels on the time to relapse or distant metastases. This was achieved using KMplot [104] which allows assessment of the relationship of 22,277 genes on the survival parameters in up to 1908 breast cancer patients. Using this resource we were able to assess the effects of these genes on relapse free survival and distant metastasis free survival as a result of Kaplan Meier plots.

Interestingly, the presence of CK1 or CK2 β had no effect on relapse free survival whereas the presence of ZIP8 reduced survival 20% in 10 years and CK2 α reduced survival 40% in 15 years. CK1 again had no effect on the production of distant metastases whereas the presence of ZIP7 or STAT3 caused a 20% reduction in survival and the presence of CK2 α or CK2 β caused a 40% decrease in survival. This data fits well with what we had found in our cell models and suggests an important role for zinc in clinical breast cancer progression. This role of zinc fits the model described in Figure 5 where zinc transporters such as ZIP7 located on intracellular membranes could increase the intracellular zinc content by emptying stores when activated by CK2. Additionally, the presence of ZnT1 actually provided a 20% increase in survival in 10 years. This would no doubt be due to the ability of ZnT1 to transport any excess zinc out of the cells [3] and help maintain the normal intracellular levels of zinc.

4.6. Zinc and Zinc Transporters in Prostate Cancer

The observed changes in zinc handling in the prostate during prostate cancer appear to be the direct opposite of what has been observed in breast cancer, namely that cellular zinc decreases during prostate cancer [105]. The normal human prostate accumulates zinc primarily by using ZIP1 to uptake zinc from the circulation and using ZIP2 and ZIP3 to maintain the zinc within the cells [106]. However, this ability to accumulate zinc in cells is lost in prostate cancer owing to the reduced expression of ZIP1, ZIP2 and ZIP3 [106]. The importance of ZIP1 to maintain high levels of zinc in prostate has been reinforced by the demonstration that ZIP1 overexpression reduces the metastatic ability of prostate cancer cells [107]. However, in both breast and prostate cancer the normal zinc homeostasis is dysregulated to the opposite direction of normal levels.

4.7. Zinc and Zinc Transporters in other Cancers

To date at least two zinc transporters have been implicated in the progression of pancreatic cancer, suggesting that what has been discovered about the ability of elevated intracellular zinc to drive cancer growth in breast cancer may have relevance to other cancer types. Pancreatic cancer growth has been inhibited by removal of ZIP4 using siRNA and this also increased the survival of mice with pancreatic cancer xenografts [108]. Investigations to discover how ZIP4 regulates pancreatic cancer has unearthed an involvement of ZIP4 in angiogenesis, invasion and metastasis pathways by causing increased expression of neuropilin-1, vascular endothelial growth factor, and matrix metalloproteases in pancreatic cancer cell lines and xenografts [109]. Another study using xenografts either over- or under- expressing ZIP4 suggested that ZIP4 overexpression caused cAMP response element-binding (CREB) induced interleukin 6 (IL-6) transcription which in turn activated STAT3, increased cyclin D1 expression and resulted in increased cell proliferation and tumor progression in pancreatic cancer [110]. Furthermore, ZIP4 has also been demonstrated to be involved in hepatocellular carcinomas by repressing apoptosis, enhancing movement through the cell cycle and increasing migration [111]. An investigation of ZIP6 in pancreatic cancer found elevated ZIP6 levels in cancer cells or tissues compared to normal with its expression correlating to tumour size and lymphatic infiltration [112]. Furthermore, removal of ZIP6 using siRNA inhibited cell proliferation and motility in vitro and tumour growth and metastasis in vivo. These effects of ZIP6 were associated with activation of Snail and loss of E-cadherin, hallmarks of induction of epithelial to mesenchymal transition [112], effects previously observed for ZIP6 in breast cancer and development [85; 88]. This data suggests a similar role for zinc and zinc transporters in pancreatic cancer to that already observed for breast cancer.

5. Conclusion and Perspectives

From the data in this chapter it appears that there is a defined role for zinc in the ability to drive cancerous cell growth. Most cancer tissues examined to date appear to have elevated intracellular zinc which, through the newly evolving zinc signaling pathways and the ability of zinc to inhibit phosphatases, would encourage activation of tyrosine kinases and their associated signaling pathways which are known to be capable of driving aggressive cell growth, proliferation and invasion. It would be a complex task to inhibit zinc in specific cancer cells and therefore any information that can be discovered about the way the different zinc transporters interact or function to increase intracellular zinc will provide the key to targeting intracellular zinc in this disease.

With respect to breast cancer, individual signaling pathways have been targeted in the clinic to prevent cancerous growth. This strategy has been flawed as the tumour cells, although reduced in growth initially, develop the use of new signaling pathways to evade this blockade which can result in more aggressive growth than that of the original tumour. However, one particularly exciting benefit of targeting zinc in these circumstances is that this approach has the potential to block multiple signaling pathways at one time, due to the ability of zinc to inhibit widespread phosphatases, and thus remove the opportunity for the cancer cells to evade this blockade. In the case of breast cancer this could be an exciting move forward by ensuring the cancer cells

continue to respond to existing anti-oestrogen drugs and fail to develop any anti-hormone resistance.

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15. Zinc in Pregnancy

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Abstract. The essentiality of zinc is highlighted in pregnancy where many fundamental processes are dependent on Zn. In addition to adequate intake during pregnancy and lactation, it is recognised that a range of pathological outcomes are associated with zinc deficiency including; birth defects, growth retardation, impaired immune function and increased susceptibility to infection, skin disorders and central nervous system dysfunction. Furthermore, our understanding of pregnancy outcomes has been advanced by the better understanding of mechanisms that underlie the altered supply of Zn to the fetus in response to the maternal exposure to toxins, infection and disease. The metal binding protein, metallothionein is important in altering Zn distribution following exposure to alcohol or infection.

Keywords. Pregnancy, zinc, fetal Zn, maternal Zn, plasma Zn, metallothionein, ethanol/alcohol, infection, lipopolysaccharide, acute phase response, Zn supplementation

Introduction

Zinc is important for a normal pregnancy. It is an essential element playing far-reaching roles in biological processes. This is highlighted in the range of pathology associated with zinc deficiency including; teratology, growth arrest in infants, abnormal nitrogen metabolism, impaired reproductive capacity, impaired immune function and increased susceptibility to infection, skin disorders and central nervous system dysfunction. The latter may be manifested by neuropsychological changes such as emotional instability, mood changes and depression [1-3].

Zn is a relatively small ion with a highly concentrated charge that uniquely allows it to cross-link and form five different coordination geometries in structural and catalytic molecules. It forms the prosthetic group, conferring activity to enzymes involved in virtually all aspects of metabolism and growth. Zn is present in hundreds of 'Zn-finger' sequences making it a key structural component of gene regulatory proteins. Regulation of protein synthesis is also exerted by a variety of Zn metalloenzymes participating in the metabolic machinery, including DNA polymerase, reverse transcriptase, RNA polymerase, tRNA synthetase, protein chain elongation factor, thymidine kinase and ribonucleases [4, 5]. These Zn-binding proteins play a pivotal role, not only in general metabolic processes (e.g. reproduction, vision, taste, immune function and cognitive behaviour), but also in the regulation of growth and development. Free Zn ions play an important role in cellular signaling and a growing

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list of molecular targets have now been identified (see Chapter 6). Zn also acts as a neurotransmitter and is selectively stored and released from neurons, specifically those that release glutamate in the cerebral cortex [6]. Thus, at the cellular level, Zn participates in protein, nucleic acid, carbohydrate and lipid metabolism, as well as the control of gene transcription and the regulation of cell proliferation, differentiation and apoptosis [2, 7]. With the variety of roles that Zn has in physiological systems, it is easy to understand why Zn is critical for fetal growth and development.

1. Adaptive Response to Zinc Utilization in Pregnancy

The importance of Zn in reproduction is reflected by the increase in the daily Zn requirement throughout pregnancy, from 2.0 mg at the beginning to 2.6 mg at the end [8]. Based upon estimates that the bioavailability of Zn from the diet is 25%, it is thought that at least 10.5 mg/Zn per day is required to meet the Zn requirements late in gestation. As women do not consistently increase their Zn intake during this period, an adaptive response in Zn uptake and/or retention was proposed to meet this increased need for Zn during pregnancy and lactation. Certainly, early studies have indicated that urinary Zn excretion is less in pregnant women with equivalent Zn intakes, however, this was not found to be the case in late pregnancy [9]. Early studies using rat intestinal loop or inverted rat intestinal sac, reported an 80% increase in duodenal Zn uptake and transfer by the end of pregnancy. This duodenal increase did not appear to be due to an overall increase in nutrient absorption as the lysine absorption was unaffected [10, 11]. An adaptive response in Zn absorption also appears to occur in humans when Zn requirements are increased during pregnancy and lactation [12, 13]. In a stable Zn isotope study conducted on women from northeast China, the fractional absorption of Zn was found to be 70% higher during the second month of lactation compared with non-lactating women on similar low Zn intakes. These women also increased their total food intake during lactation thereby increasing their overall intake of Zn by 50%. Fecal endogenous Zn losses were also lower [13]. A similar finding was reported in a longitudinal study that showed that fractional Zn absorption was increased during lactation but was not significantly different in the period before conception and the end of the second trimester in women on normal Zn intakes [14]. However, a second study, conducted on Brazilian women with low Zn intakes, found that the fractional Zn absorption increased from 29 to 43% from the beginning to the end of pregnancy and remained at this level during lactation. Here, the increase in fractional Zn absorption was inversely proportional to plasma Zn concentration [15]. In this latter study, two exchangeable extra vascular Zn pools were identified based upon a three compartment model of 24 h Zn kinetics. The model predicts that plasma Zn exchanged bidirectionally with two distinct compartments. The first, a rapid turnover compartment where the fractional rate of Zn transfer was highest at the end of pregnancy and the second, a slow turnover compartment where the Zn flux was highest at the end of lactation. It was proposed that different Zn kinetics suggested that different processes might be involved in meeting the Zn requirements for fetal growth and milk production [15]. Clearly, more studies are required in order to understand the compensatory mechanisms that interact to improve Zn uptake and retention as well as clarifying the signaling pathways that control this adaptive response to maintain Zn homeostasis in pregnancy and lactation.

2. Zinc in Human Milk

Maternal Zn absorption is increased and excretion decreased late in pregnancy and during lactation. In the first 6 months of life infants require 2 mg of Zn per day. The concentration of Zn in human milk declines rapidly after birth; being 4 mg/L at 2 weeks, 2 mg/L at 2 months and 1.2 mg/L at 6 months [16, 17] compared with cows milk which ranges from 3-5 mg Zn/L. However, the bioavailability of Zn from human milk is much greater than from cows milk, which is possibly related to the enhanced digestibility of the protein content of human milk [18].

Zn is predominantly bound to citrate in human milk [19]. The mammary gland facilitates the transfer of large amounts of Zn from the maternal circulation and maintains milk Zn concentration even when the dietary Zn intake is marginal, suggesting that homeostatic control of Zn occurs within the mammary gland [20]. While little is known about this process, the detection of Zip3 on the plasma membrane of mammary epithelial cells, implicates it in the uptake of Zn from the maternal circulation. Zip3 is recognised as a tissue specific member of the Zip family and is mainly found in tissues with a high Zn need. The localization and expression of ZnT1, ZnT2 and ZnT4 in mammary epithelial cells suggest a significant role in the transfer of Zn into milk. These effluxers are found on the luminal surface with the staining greatest early in lactation compared with late. Peak Zip3 and ZnT4 expression levels also occur in the mammary gland early in lactation. It is argued that these changes explain the decline in Zn milk levels that occur as lactation progresses [21]. Investigations with cultured mouse mammary epithelial cells provide evidence that the lactogenic hormone, prolactin, may influence the Zn content of milk. Prolactin increased the serosal-to-lumina Zn transport, changed the expression of Zip3 and ZnT4 and caused the recruitment of Zip3 to the serosal surface and ZnT4 to a perinuclear location. The authors have speculated that movement may involve prolactin-mediated phosphorylation of these transporters [21, 22].

It is now evident from X-Ray Fluorescence Microscopy studies on mouse mammary epithelial cells, that Zn imported from the maternal circulation is concentrated within perinuclear vesicles before being secreted in milk. Large discreet pools of Zn were identified in mitochondria and the Golgi apparatus consistent with findings in the prostate gland before it is taken up by vesicles for secretion in seminal fluid. However, in the mammary gland, only the pool of Zn in the Golgi apparatus appears to be labile and capable of being mobilized for secretion of Zn in milk. While Zip7 and Zip9 have been implicated in Zn trafficking in Golgi apparatus [23], the complex integration of Zip family transporters with others involved in the regulation of the influx and secretion of Zn in the mammary gland remains to be elucidated [24].

A recessive point mutation that occurred spontaneously in C57BL/6 mice has been named the lethal milk (*lm*) mouse allele as offspring, irrespective of genotype, raised on milk from homozygous mutant dams die before weaning from symptoms of systemic Zn deficiency [25]. Mutant pups survive if fostered on the milk of normal mice which contains approximately 34% more Zn than that of the mutant homozygote dam [26]. The defect has been mapped to the SLC30A4 gene on chromosome 2 which encodes for the vesicular Zn efflux transporter, ZnT4 [27], resulting in premature protein termination. While a functional ZnT4 is required to fully concentrate the amount of Zn in milk, the fact that measurable amounts of Zn are determined in milk from *lm/lm* mice clearly implicates other transporters in the Zn secretory process. Non-mammary gland defects have also been found in *lm/lm* mice that include a change

in the expression of transporters involved in intestinal Zn trafficking and the development of some symptoms of Zn deficiency late in adult life [28, 29].

A Zn deficiency disorder in preterm human babies is also known to be caused by low Zn levels in their mother's milk [30-32]. These infants are normally of gestational age between 27-33 weeks and develop Acrodermatitis Enteropathica-like symptoms, but show a normal capacity to absorb Zn in the gut. Lineage mapping indicates that the disorder is hereditary and carried by the mother who appears to be unable to successfully concentrate Zn in her milk. It was hoped that a defect in SLC30A4 gene might, in part, explain the low level of Zn secretion in breast milk. However, when hZnT4 levels were determined in fibroblasts, lymphoblasts and buccal cells from mother's of the infants with Zn deficiency, they were found to be the same as in controls [33]. In a review, Ackland and Michalczuk reported decreased expression of hZnT5 and hZnT6 mRNA in fibroblasts and lymphocytes from two patients with mammary Zn secretion disorder and further suggested that these transporters may putatively be involved in the aetiology of the human disorder [34].

3. Zinc Deficiency

Animal studies have been valuable in demonstrating the link between fetal Zn deficiency and teratology [35-39]. Long-term Zn deficiency in rats fed a diet containing less than 0.5 mg/kg of Zn throughout pregnancy (compared with 35 mg Zn /kg control) impairs reproduction and decreases fetal body weight, with 90% of the fetuses demonstrating gross malformations affecting every organ system [40]. Short-term Zn deficiency is also teratogenic, but with altered incidences of abnormalities depending upon the timing of the deficiency relative to the stage of development. In rodents, a single day on a Zn deficient diet can be sufficient to reduce the maternal plasma Zn levels in a pregnant dam by 30% [39, 41]. In early pregnancy, Zn deficiency is associated with defects of the head region, including the eyes, facial structures and brain. Later in pregnancy, Zn deficiency results in a more frequent incidence of skeletal malformations and defects in the urogenital system and tail [39]. A number of studies have reported that Zn deprivation during pregnancy and lactation can result in poor fetal activity, newborn motor development, learning and long-term, short-term and working memory in adult offspring [38, 42-47].

3.1. Acrodermatitis Enteropathica

Acrodermatitis Enteropathica (AE) is a rare (1/500,000 children) autosomal recessive disorder that results in insufficient Zn uptake by the duodenum and jejunum [48]. Infants with AE often present with acral dermatitis, alopecia and diarrhea. If left untreated, symptoms progress to include growth arrest, reduced immune function and neuropsychological disturbances [49]. While the disease can be fatal, early diagnosis and therapy with Zn returns normal function which can be maintained over a lifetime providing the patient is compliant with therapy [50]. Diagnosis is likely to be made when symptoms are associated with a low serum Zn level of less than 7.8 µmol/L. Symptoms of AE can arise in infants within days after birth if bottle-fed, and within weeks after weaning when breast-fed, the discrepancy thought to be due to increased bioavailability of Zn from human milk compared with cows milk [49].

The mutation has been localized to chromosome 8 and the defective gene identified as SLC39A4, which encodes a Zn transporter protein, Zip4, responsible for the uptake of Zn from the lumen into the enterocyte. Under normal circumstances, the expression of SLC39A mRNA is responsive to Zn and in mice is up-regulated during Zn deficiency and down-regulated by Zn supplementation [51]. Zip4 accumulation in the apical membrane is also regulated through a Zn-dependent ubiquitination of a histidine-rich segment that results in endosomal degradation when Zn is plentiful. [52, 53]. The fetuses of homozygous Zip4 knockout mouse (KO) do not proceed past gestational day 8-10 [54], which importantly, is the critical period of organogenesis. The deformities seen in these fetuses are consistent with those seen with Zn deficiency. Interestingly, orally gavaged Zn or Zn given by intraperitoneal injection was ineffective in rescuing the Zip4 KO fetuses. Heterozygous Zip4 KO mice were sensitive to Zn deficiency and showed growth retardation and physical abnormalities [54].

Many of the mutations identified in the AE gene appear to hinder Zip4 accumulation on the apical surface by causing either misfolding or mislocation of the protein that, putatively, has eight transmembrane domains, or a defect in the Zn sensing mechanism in the regulation of mZip trafficking [52, 55]. Other variants appear to alter the Zn-responsiveness of the endosomal breakdown process. As mutations of the SLC39A gene are not always identified in patients showing symptoms of AE, a second AE gene has been postulated [56, 57]. In addition, there is also an acquired form where patients display the same triad of AE-like symptoms and this may result from poor dietary Zn intake due to low Zn bioavailability caused by dietary or iatrogenic factors, or intestinal malabsorption syndromes, particularly when coupled with increased physiological demands for Zn during pregnancy and lactation. Zn replacement therapy (typically around 3 mg Zn/kg/d) which allows Zn to be absorbed by paracellular pathways results in very rapid clinical improvement, typically within days or weeks and often before serum Zn levels improve [49].

4. Zinc Transporters

Our understanding of how cells and organelles transport Zn has seen major advances in the last decade, through the application of molecular techniques and the production of transgenically modified cells and animals. Lichten and Cousins [58] have recently published an extensive review of this area and these are further dealt with comprehensively in Chapter 8 of this book. The two groups of proteins which act to transport Zn are the ZnT family of transporters, which function to decrease intracellular Zn levels, and the Zip family, which do the reverse by increasing intracellular Zn levels. Within cells, ZnTs increase Zn movement into organelles such as lysosomes, vesicles and secretory granules, while Zip proteins transport Zn from the lumen of organelles into the cytoplasm. These proteins are essential for Zn homeostasis, including the primary functions intestinal Zn transport (ZnT1, Zip4, Zip5), renal Zn re-absorption (ZnT1, Zip10) and pancreatic release of Zn (ZnT1, ZnT2, Zip5). The example of Zip4 protein is particularly instructive in relation to Zn and the fetus due to its central role in AE.

In regard to pregnancy, the change in expression from maternal to fetal hepatic MT would also appear to be coordinated with a number of Zn transporters that potentially mediate the transfer of Zn between mother and fetus in late gestation [59]. In that study, expression of placental ZnT1, ZnT4, ZnT5, Zip1 and MT were found to be

responsive to maternal dietary Zn. Mouse embryo's with homozygous mutations of the ZnT1 gene die in utero around GD 8, indicating that this effluxer plays a primary role in the maternal to embryonic transfer of Zn [60]. The ZnT gene family has also been found to respond independently during lactation in the mouse, presumably to provide adequate Zn nutrition to the neonate [61].

Zip1 and Zip3 expression has been detected in mouse pre-implantation embryo's suggesting an early role in the acquisition of Zn into the embryo. However, studies on Zip1 and/or Zip3 knockout mice indicate they are not essential for a successful pregnancy when maternal Zn supply is adequate, but are more sensitive to teratogenicity when maternal dietary Zn is severely limited [62]. The role of Zip1 and Zip3 in pregnancy is unclear but may be surmised from in vitro studies using HEK 293 cells that were stably transfected with the mouse Zip genes and then cultured in Zn deficient medium. In those studies, mZip1 and mZip3 accumulated on the plasma membrane as a result of a decrease in Zn-responsive endocytosis, which causes an increase in Zn uptake by the cells. It was proposed that this form of post-translational control may regulate Zn homeostasis in many other cell types where Zip1 and Zip3 are co-located [55]. That these transporters are functionally redundant when maternal Zn supply is adequate, suggests that there are other compensatory mechanisms yet to be discovered. Zip3 has also been found in mammary gland epithelial cells where it is thought to be involved in the re-uptake of Zn from milk previously secreted into the alveolar lumen. It has been argued that this role may have evolved to scavenge Zn for apoptotic processes that are needed in the mammary gland as it reduces in size during weaning [22]. Clearly, a future challenge will be to understand the signaling processes that control the expression of Zn binding and transporter proteins and to determine how they interact to regulate maternal-fetal Zn exchange at critical periods of gestation.

5. Maternal and Fetal Morbidity and Serum Zinc

There is considerable evidence linking low serum Zn status with abnormal fetal outcomes [63-65]. Serum Zn declines progressively during pregnancy in relation to blood volume expansion [66]. This can be further decreased by decreased dietary Zn intake, intercurrent disease (infection, inflammation), toxins or genetic factors. A clear example of a genetic influence can be seen in the reported increase in congenital malformations of AE patients which can be protected by Zn supplementation [67]. However, infection, inflammatory disease and alcohol intake present the most significant risk for pregnant women. Blood Zn in pregnant women is 15-35% lower in the first trimester than in non-pregnant women coinciding with the period of maximal susceptibility to teratogens during organogenesis [68]. It is in these areas where our understanding of underlying mechanisms in relation to altered Zn supply to the fetus has seen significant advances, in particular, the role of the Zn binding protein metallothionein (MT).

6. Ontogeny of the Zinc Binding Protein Metallothionein during Pregnancy

MT is not essential for normal pregnancy [69], however, it nonetheless plays an important role during gestation, particularly in protecting the fetus against maternal Zn deficiency. This is evident from studies using MT-knockout mice that have been found

to be more sensitive to teratogenic events from maternal Zn deficiency [70], whereas, transgenic mice that overexpress MT-1 are found to be resistant to this challenge [71]. Thus, MT appears to have functional redundancy in Zn replete dams but acts as a biological buffer in Zn deficient states by providing a source of labile Zn that maintains normal fetal growth and development when the mother's dietary Zn supply is restricted. Temporal elevations in liver MT mRNA and protein levels have been reported during the perinatal period in fetal and newborn rats [72-75] and mice [76, 77]. In rodents, fetal liver MT levels increase in late gestation, attaining dramatically high levels at birth and thereafter decreasing towards adult levels around weaning (PD 21) [78, 79]. This peak in liver MT in the newborn is at least 15-fold higher than that which is induced in the mother's liver throughout gestation and would appear to be an adaptive response to sequester Zn to the fetus late in pregnancy [78]. Maternal liver MT peaks on GD 15 before subsequently falling towards non-pregnant levels close to parturition [78] (Figure 1). These changes in MT protein parallel changes in MT mRNA expression that are thought to be regulated by maternal corticosterone levels and uterus-derived IL-6, the concentrations of which, rise and fall over a similar time course during gestation [77, 80]. It can be surmised that perinatal fluctuations in MT are required to provide a source of exchangeable Zn which is necessary for coping with the extreme metabolic demands of the dam and its fetus, and/or later in gestation for placental transfer when the fetal liver is able to cope with regulating its own Zn homeostasis. In rodents, the rise in maternal liver MT during pregnancy is associated with a fall in plasma Zn that is not unlike that in human pregnancy, where plasma Zn concentrations decline in early pregnancy and continue to remain low until term reaching levels 35 % below that in non-pregnant women [81].

7. Implication of an Inappropriate Induction of Metallothionein in Pregnancy

Investigators using isolated term human placenta, have demonstrated that placental Zn transport is bidirectional, requires ligand binding, does not proceed against a concentration gradient of Zn and occurs at a slow rate that is only 6% of that of a freely diffusible marker [82]. Since the fetus does not stockpile Zn, an adequate maternal supply is required to maintain normal developmental processes. The maternal plasma is the conduit of Zn for placental/fetal interchange, thus, it follows that inappropriately low concentrations can affect the maternal-to-fetal Zn gradient so that it opposes the movement of Zn into the fetus. Such has been found to be the case in rodents following exposure to a range of xenobiotics in pregnancy. Daston and coworkers were the first to demonstrate that urethane, when injected into pregnant rats on GD 11, significantly induced maternal liver MT, decreased maternal plasma Zn concentrations by 30%, and inhibited the transfer of ^{65}Zn into the fetus by 50%. When the GD 18 fetuses were examined they exhibited decreased weight and delayed skeletal ossification [83]. A range of other toxicants with different pharmacological actions, such as α -hederin, TNF- α , 2-ethylhexanoic acid, arsenic, and alcohol, were later demonstrated to have similar effects on MT, Zn distribution and fetal outcome [84-87].

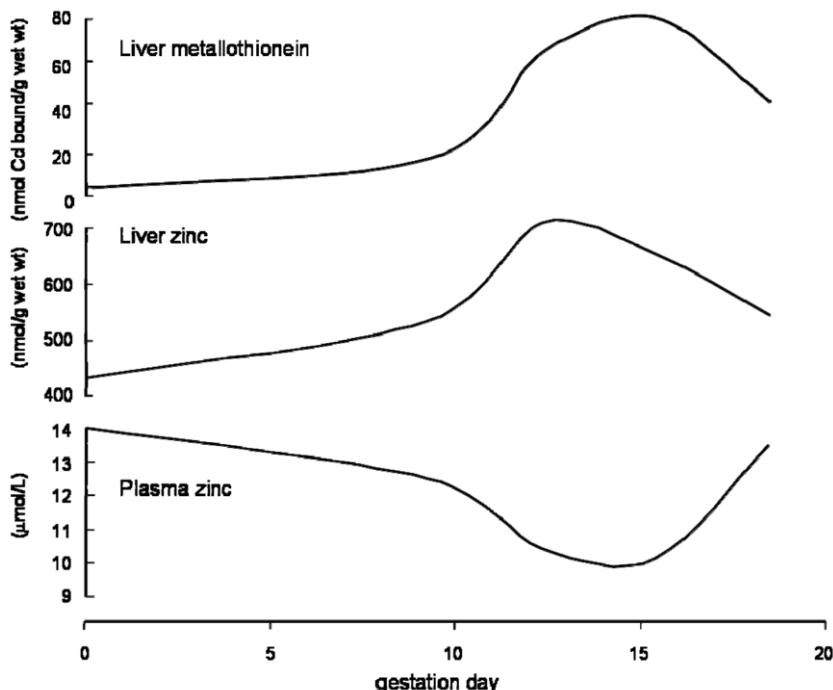


Figure 1. Typical changes in liver metallothionein, liver Zn and plasma Zn during gestation in the C57BL/6 mouse dam. Parturition occurs on day 21. The data taken from Coyle et al., 2009 [78] shows the increase in metallothionein that occurs in the mother's liver which peaks on gestation day 15 and falls towards non-pregnant levels by parturition. The gestational rise in metallothionein is concomitant with an increase in liver Zn and a fall in plasma Zn.

Our group extended these studies in the C57BL/6 mouse with investigations on the prenatal administration of ethanol or lipopolysaccharide (LPS) on pregnancy outcome. In our alcohol studies, we demonstrated that a single binge resulting in blood alcohol levels of 0.2-0.3% over 8h, when administered at the beginning of organogenesis (GD 8), increased the maternal liver MT by 20-fold and decreased maternal plasma Zn concentrations by up to 65% within 16h [88]. In GD 12 fetuses, an alcohol-mediated decrease in maternal plasma Zn was found to markedly impair ^{65}Zn transfer to the fetus and cause a 20% reduction in total fetal Zn content, 7h following alcohol [89]. When we administered LPS, which causes an acute inflammatory response in the mother, on GD 8, we again found a marked induction of maternal MT that was accompanied by a severe fall in plasma Zn. Alcohol or LPS administration on GD 8 was associated with significant increase in teratogenicity that was strikingly similar in phenotype to that reported in studies where pregnant dams had been made Zn deficient. These abnormal pregnancy outcomes include low birth weight, microphthalmia, anophthalmia, exencephaly, clefts of the lip and palate, major skeletal defects and cognitive deficits in offspring [37, 90-102].

These findings, although not conclusive, are highly suggestive of a common mechanism that causes teratogenicity from various toxicants that is potentiated during the period of major organogenesis (day 7-14 of gestation in the rodent), a time of rapid development and differentiation. Accumulating evidence suggests that this mechanism

involves maternal hepatic MT induction that is associated with fetal Zn deficiency (Figure 2). The hypothesis is that when maternal hepatic MT is induced by various xenobiotics, Zn is sequestered from the maternal circulation into the liver to bind to cysteine residues on the newly synthesized apo-MT. This movement of Zn results in depletion of albumin-bound Zn which is the main exchangeable pool of Zn in plasma. The fall in plasma Zn concentration lowers the maternal-to-fetal Zn gradient and the net effect is that fetal Zn uptake is compromised. It would be predicted that teratogenicity will depend upon the severity and length of the fetal Zn deficiency, and when it occurs relative to vulnerable windows of gestation.

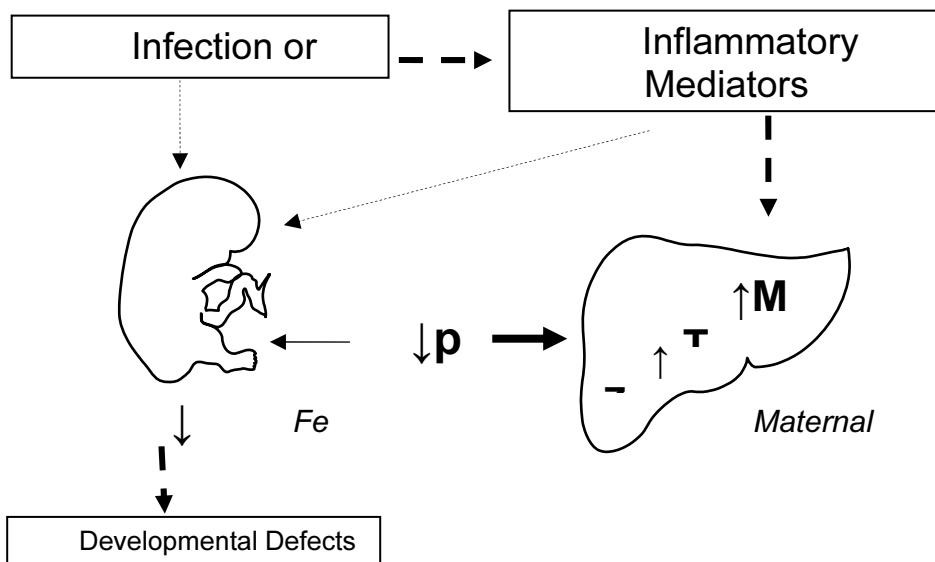


Figure 2. Changes in maternal-fetal Zn homeostasis that occur as a result of a systemic infection or exposure to ethanol in pregnancy which have the potential to cause developmental defects. Ethanol or various infectious agents may affect the fetus directly or indirectly via inflammatory mediators; however, their overall contribution to teratogenicity is unclear. These mediators can also induce metallothionein (MT), a Zn-binding protein predominantly induced in the liver. The induction of MT causes the sequestering of exchangeable Zn to the mother's liver which in turn, results in a decrease in plasma Zn concentration. The fetus does not stockpile Zn but relies on a constant maternal supply and as placental Zn transfer is bidirectional and does not proceed against a concentration gradient, a low maternal plasma Zn impairs maternal-to-fetal Zn transfer causing a short-term depletion of exchangeable Zn in the fetus. As Zn is required for plethora of functions, a transient deficiency of Zn may interrupt or change the physiological processes programmed for that gestational age.

Perhaps the most compelling evidence linking the induction of MT with teratogenicity comes from our findings in MT-knockout that were derived by Michalska and Choo [103]. These mice lack the two inducible MT genes, MT-1 and MT-2. In our studies where we gave alcohol or LPS to wild type or MT-knockout mice on GD 8, we observed a significant increase in birth abnormalities in wild type mice but not MT-knockout mice. The MT-knockout mice were resistant to alcohol and LPS with levels of teratogenicity either the same or lower than those in saline-treated controls. As expected, the hypozincemia which followed LPS or ethanol administration in wild type mice was not observed in MT-knockout mice. We have further conducted studies in wild type mice where we have replenished the maternal Zn

either with subcutaneous Zn injection at the time of the ethanol or LPS challenge, or as a dietary Zn supplement, throughout pregnancy. Both methods of Zn treatment were found to prevent the depression of plasma Zn concentration associated with MT induction in the liver. Most importantly, both forms of Zn treatment were found to be effective in preventing birth defects [91-93, 102]. These findings indicate that the concentration of Zn in the mother's blood is an important determinant in the aetiology of birth defects caused by these ethanol or LPS and that these negative birth outcomes can be prevented by improving the mother's Zn status.

Our C57BL/6 mouse model shows the full range of birth defects and is particularly sensitive to neurological change [90-92, 101, 102, 104]. In addition to determining a specific link between alcohol or LPS administration and teratogenicity, we have shown that memory deficits are also apparent in the wildtype offspring that appear to have no visible birth defects. These memory deficits were detected in adult offspring that were randomly selected from litters produced by dams treated with alcohol or LPS on GD 8 when tested in a water cross maze that assesses spatial learning and memory, and an object recognition task that measures a non-spatial form of short-term memory [101, 104]. Most interestingly, offspring from dam's given ethanol, but were fed a Zn supplemented diet throughout pregnancy, had normal scores, demonstrating that dietary Zn supplementation protects against the ethanol and LPS-related cognitive deficits [104]. We also found that Zn treatment prevented the increase in post-natal morbidity associated with prenatal alcohol exposure. Cumulative postnatal mortality was significantly higher in offspring exposed to alcohol alone (35% deaths) compared to control (10% deaths) or alcohol and dietary Zn supplementation (12% deaths) [102]. Taken together, our findings indicate that a positive maternal Zn status may help ameliorate much of the teratogenicity, postnatal mortality and cognitive impairments associated with acute alcohol exposure or an acute inflammatory response in pregnancy.

Although all the evidence supporting an MT-mediated mechanism of teratogenicity is derived from animal models, there are indicators that it may apply to several human developmental disorders. In this regard, there is a striking similarity in the spectrum of physical and cognitive abnormalities found in rodents prenatally exposed to alcohol and those in children with Fetal Alcohol Syndrome (FAS) and Alcohol Related Neurodevelopmental Disorders (ARND). In addition, it has long been known that alcoholic mothers have significantly lower plasma Zn levels than non-alcoholic women and that an inverse relationship occurs between maternal plasma Zn levels and expression of FAS [105]. While Zn limitation of less than one day is sufficient to cause teratogenicity and neurodevelopmental anomalies in mice, this period can only be surmised in humans. As the half-life of MT is approximately 20 hours, the critical period in women could be up to 48 hours. As obvious ethical considerations preclude the use of alcohol in pregnant volunteers, further investigations will need to be conducted in primates or large animal models where developmental milestones and the length of gestation can be more closely mimicked.

Induction of hepatic MT as a result of infection and chronic disease is a well recognised cause of hypozincæmia in humans indicating a putative involvement in the aetiology of developmental abnormalities associated with prenatal infections. Epidemiological studies have associated prenatal infections with the pathogenesis of cerebral palsy [106, 107], schizophrenia [108-112], non-genetic forms of autism [113], and mental retardation [114]. This association is strengthened by findings from animal studies where both bacterial and viral infections *in utero* have been shown to cause a

spectrum of neuropathological and behavioural abnormalities in offspring (reviewed by [115, 116]). Although it remains unclear how such a broad range of infectious agents might trigger neurodevelopment anomalies, accumulating evidence suggests that the damage may be due to the maternal immune response rather than the infectious agent itself [115, 116]. While pro-inflammatory cytokines (IL-6, IL-1- β and TNF- α) have been investigated as putative mediators of brain damage, their causative role still remains unclear [117-120]. A comprehensive review of mechanisms by which the maternal immune response to prenatal infection can hinder neurodevelopment has highlighted the growing interest in this field and deficiencies in knowledge [115]. It is known, however, that the Zn transporter Zip14 is involved in hypozincæmia related to infection and inflammation. In a study using IL-6 KO mice, it was demonstrated that IL-6 was a primary inducer of Zip14 and MT in the liver [121]. The LPS-induction of MT was not completely abolished in the IL-6 KO mice, suggesting that other inflammatory mediators play a role in this event. In this regard, the promoter region on the MT-gene is activated by a range of effectors including reactive oxygen species, inflammatory cytokines (IL-1 β , TNF- α and IL-6), corticosteroids and xenobiotics (including alcohol). In addition, many of these effectors work in combination to activate nuclear factors that cause a synergistic response on MT gene transcription [122-124]. However, paramount to understanding how prenatal infection putatively affects fetal Zn uptake is a requirement for further studies on the interrelationship between metal binding proteins and Zn transporters involved in sequestering of Zn in the mother's liver and involved in placental transport.

A further consideration in regard to MT induction by toxicants is whether an equivalent level of effector-mediated induction causes a similar deficit in fetal Zn as pregnancy progresses. As there is a normal physiological increase in maternal MT that peaks in mid-to-late gestation, it might be predicted that this would limit the capacity for further induction of MT by various effectors. This has not proved to be the case with LPS or ethanol, where significant levels of MT induction have been found throughout gestation (Figure 3). Whether the impact of this induction on fetal Zn levels in mid-to-late pregnancy is as devastating to the fetus as that in early pregnancy [89] warrants further investigation. It would seem likely that the embryo would be more vulnerable to changes in Zn in early pregnancy when histotrophic nutrition, via the yolk sac and uterine glands, requires phagocytotic processes that are potentially more susceptible to the protein-bound Zn concentration in the extracellular fluids of the endometrium and uterine glands [125]. Later in pregnancy when placenta is functional and the fetus has developed its own homeostatic mechanisms for Zn, temporary reductions in maternal blood Zn caused by inappropriate MT induction might have less impact particularly if dietary Zn is plentiful. While the transition from histotrophic nutrition to a functional placenta occurs earlier in humans than in rodents [126], it nonetheless is thought to be an important source of nutrients throughout organogenesis in the first trimester [127].

Although the relevance of inappropriate MT induction to human teratogenicity remains unclear, several predictions can be made from this mechanism. The first is that any inducer of MT during pregnancy has the potential to cause whole-body Zn redistribution and thereby be detrimental to fetal development. The second is that exposure to combinations of MT-effectors could have additive or synergistic impact on fetal Zn status. It is assumed that the mother's Zn status at the time of insult would be critically important to fetal wellbeing as poor maternal Zn nutrition would most likely exacerbate any effector-mediated MT response on Zn homeostasis in the fetus. In this

regard, it is perhaps not a coincidence that FAS and ARND are over represented in some indigenous communities, where substance abuse, chronic infections and poor nutrition are endemic.

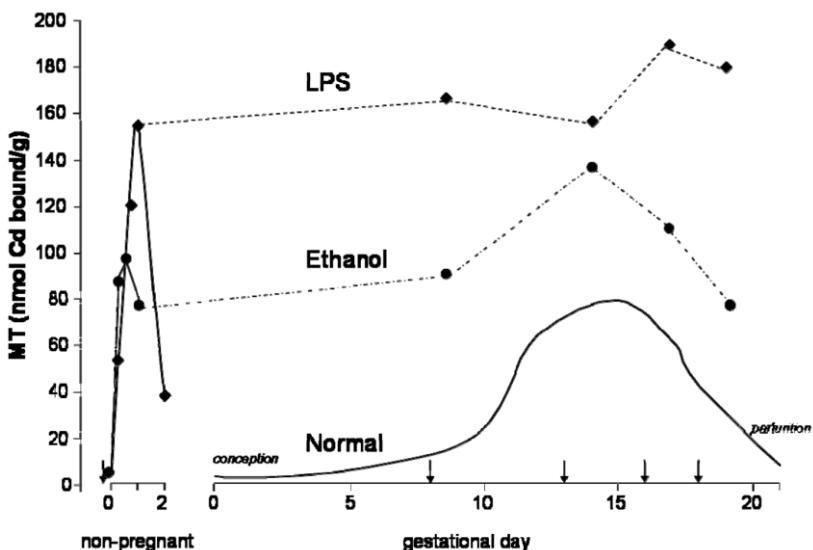


Figure 3. The effect of acute injections of LPS or ethanol on maternal liver metallothionein levels during pregnancy. Pregnant and non-pregnant C57BL6 mice were administered lipopolysaccharide (LPS; 0.5 µg/g, injected s.c.) or ethanol (two i.p. of 25% v/v; 0.015 ml/g injections, 4h apart). The treatments were administered on gestational days 8, 13, 16 or 18 as shown by the arrows. Shown is the time course of MT induction in non-pregnant mice for each treatment and the MT concentration, 24h after LPS (closed diamond) or ethanol (closed circle) in pregnant mice. The normal gestational changes in MT are also shown (closed line). At all time points, MT was markedly increased above gestational levels, matching levels seen in non-pregnant mice, after exposure to ethanol and LPS.

8. Zinc Supplementation

There is little convincing evidence supporting the use of dietary Zn supplementation to improve pregnancy outcome in healthy women. A major review of 17 randomized control trials (RCTs) was recently taken from the Cochrane Pregnancy and Chilbirth Group's Trials Register and the effect of Zn supplementation during pregnancy, labour and birth was determined on maternal and fetal health and well being [128]. These trials were conducted over three decade's in 10 countries and assessed the pregnancy of nearly 9000 women who were recruited on the basis that they had no underlying illness. Thirteen trials contained subgroups of women of low income status that were suspected of being Zn deficient as a result of being malnourished. The daily supplement of Zn in these studies was between 15 to 44 mg with the minimum duration of supplementation during gestation being 26 weeks. The only significant finding was a 14% reduction in the number of preterm births in women taking Zn and this was primarily due to a subset of studies conducted in Bangladesh, Nepal and Peru where women from low income families were more likely to undernourished and perhaps suffer from chronic infections. However, the consensus was that the overall benefit was not considered compelling enough to support the need for supplementation of Zn in isolation from

other micronutrients. It was concluded that improving the overall nutritional status (including Zn) of pregnant women, particularly in low-income regions, would be more beneficial.

The antenatal use of micronutrient supplements is supported by UNICEF/WHO particularly in developing countries where there is a high prevalence of malnutrition. In a randomized control trial conducted in rural Nepal, iron and folic acid were found to be the key ingredients amongst other micronutrients, including Zn, which reduced incidence of low birth weight, while folic acid alone was identified as reducing the mortality in the first 3 months of life [129]. Follow-up studies on these Nepalese children have found that maternal iron-folic acid supplementation reduced mortality among these children by 31% between birth and age 7 [130]. WHO recommends that all pregnant women in areas of high prevalence of malnutrition receive folic acid and iron supplementation to prevent anemia [131]. However, it is still debatable whether added Zn would improve any maternal or infant outcome. The potential benefits and adverse effects of adding Zn to prenatal supplements containing iron and folic acids has recently been reviewed on 22 different randomized control trials conducted over the last 30 years [132]. Many of these trials overlapped with those used by Mahomed and coworkers [128] were from the Cochrane database. Again the study was unable to find a clear benefit of taking Zn supplements above those of taking folic acid and iron during gestation. Many of the trials, however, reported conflicting findings and were limited in sample size or uniform methodology. The review endorsed the need for more robust studies on Zn supplementation and in particular in undernourished or low weight women from lower-income countries. In addition, it was recommended that investigations were warranted on the benefits and risks of taking Zn supplements prior to conception and/or throughout gestation and/or lactation on maternal and fetal morbidity with follow-up studies in early childhood to address its impact on growth and development. A recent report of a follow-up study of school-age children born to women receiving Zn supplementation during pregnancy in rural Nepal found that Zn above folic acid and iron supplementation resulted in a modest increase in height and a reduction in peripheral adiposity [133]. The authors posed the question as to whether these benefits could be sustained in Nepal and in other countries that are undergoing a Westernization of diet.

While there is little support to supplement Zn in the diet of healthy pregnant women, WHO recommends supplementation of Zn in children, particularly those in developing countries likely to be at high risk of contracting diarrhea [131, 134]. This support comes from studies demonstrating an 18% reduction in the incidence, and a 25% reduction in the prevalence, of diarrhea in infants under 5y of age [135]. Evidence is accumulating that these benefits may be gained by the earlier intervention during pregnancy. In a study conducted in Bangladesh, infants of mothers who received Zn (30mg) daily from 12 to 16 weeks of gestation until delivery had a reduced risk of acute diarrhoea, dysentery and impetigo compared to placebo treated controls. However, the benefit was found in low birthweight infants and not those with normal birthweight. [136]. In a postnatal follow-up study in Lima, Peru, of 421 infants born to women enrolled in a double-blinded, randomized control trial, significant benefits were noted in infant offspring between 6-12 months of life. Infants whose mothers were supplemented with Zn (15 mg) daily during pregnancy, had a reduced likelihood of experiencing an episode of diarrhea of longer than a week and reduced longitudinal prevalence of acute diarrhea and scabies. As the presence of breast milk did not alter the magnitude of the effect of Zn on morbidity, it was argued that Zn may have a direct

influence on immunity in utero [137]. Less compelling is the evidence that Zn supplementation may also prevent the incidence and prevalence of childhood pneumonia [135, 138, 139]. A recent review on eight randomized trials covering nearly 12,000 participants, showed overall that Zn supplementation (5-20mg/d) to infants for up to a year did not prevent the occurrence of pneumonia nor was there any therapeutic improvement found when adding Zn to antibiotic therapy. However, it was argued that these results may be confounded because of the relatively small number of studies reporting on each of the specific health outcomes [140].

9. Conclusion and Perspectives

Appropriate Zn nutrition is required for healthy pregnancy and successful birth outcomes. However, even when Zn nutrition is adequate, accumulating evidence from animal studies suggests that the transfer of Zn from mother to fetus can be impeded by the maternal immune response. Studies with various inflammogens that induce maternal hepatic MT and hypozincaemia, demonstrate a clear link between fetal Zn deficiency, teratogenicity and neurodevelopmental anomalies in offspring. We are now at a point in this field where experiments are needed to validate whether a transient change in whole-body Zn redistribution, caused by MT induction, can interrupt fetal development in a higher order species that has a longer gestational time. Moreover, the timing of insult relative to the stage of gestation also needs to be addressed. Does induction of MT late in pregnancy result in maternal hypozincaemia and can this influence fetal Zn supply? One might speculate that a fully developed placenta would employ mechanisms to maintain Zn homeostasis in the fetus that would regulate acute variations in maternal Zn supply. Testing this premise in late pregnancy is, however, limited by a paucity of appropriate histochemical markers of neurodevelopmental damage that are clearly associated with adverse behavioural and cognitive outcomes in offspring.

The fact that in animal models, Zn supplementation throughout pregnancy ameliorates teratogenicity and neurodevelopmental anomalies associated with prenatal activation of the maternal immune response, does not necessarily imply that Zn supplementation would benefit humans to improve birth outcomes. Indeed, the overall findings of multiple randomized control trials using strict criteria and low methodological bias has provided little support for this premise [128]. However, most of these trials were conducted on healthy individuals where women with complications such as systemic infections were not recruited in order to prevent confounding factors influencing the outcome. With hindsight, one might speculate that these women with prenatal infections might have received the greatest benefit from antenatal Zn supplementation on their pregnancy outcome. To our knowledge, such studies have not been performed. The efficacy of maintaining a normal or high level of plasma Zn during a pro-inflammatory response must also be questioned, as it presumes that hypozincaemia is not important in the overall immune response. This may not be the case as there is growing evidence that Zn is important in signal transduction pathways that encompass cells of the immune response. The role of hepatic MT induction and hypozincaemia in the immune response therefore needs to be clarified.

The translation of findings from animal experiments to humans is likely to pose many practical and ethical problems. Clearly, it is not acceptable or ethical to place the fetus at risk from a maternal exposure. Prospective trials on volunteers may be limited

in their ability to recruit sufficient individuals that subsequently succumb to a prenatal infection or inadvertently are exposed to another form of prenatal insult. Retrospective clinical trials will most likely be constrained by variables including; low number of volunteers with a common type of prenatal exposure, time of gestation insult, the status of the mother's nutrition at the time of the insult, the duration of supplementation and amount of Zn, whether other micronutrients are taken and the birth outcomes that are measured. In addition, although Zn supplementation of between 15-45mg/d appears to be safe during human pregnancy, this has not been adequately investigated. While these levels are tolerated and do not appear to interfere with the bioavailability of other micronutrients, an overall understanding putative long-term effects of taking Zn supplements is warranted.

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16. Zinc and Ageing

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Abstract. Ageing is an inevitable biological process with gradual and spontaneous biochemical and physiological changes and increased susceptibility to diseases. Nutritional zinc may remodel these change with subsequent healthy ageing, because zinc improves the inflammatory/immune response as shown by “in vitro” and “in vivo” studies. However, in ageing, the zinc daily dietary intake is reduced than that one recommended by the RDA. Many causes can be involved: among them, inadequate mastication, psychosocial factors, drugs interactions, altered cellular processes [zinc transporters and Metallothioneins (MT)]. These processes are very relevant because the intracellular zinc homeostasis is regulated by buffering MT and zinc transporters assigning to zinc a role of “second messenger”. Physiological zinc supplementation in elderly improves these functions with however contradictory data. Therefore, the choice of old subjects for zinc supplementation has to be better considered in relation to the specific genetic background of MT and IL-6, because the latter is involved both in MTmRNA and in intracellular zinc homeostasis. The genetic variations of IL-6 -174G/C locus when associated with those ones of MT1A +647A/C locus are useful tools for the choice of old people for zinc supplementation because improving the inflammatory/immune response, suggesting the relevance of zinc-MT gene interaction for healthy ageing and longevity.

Keywords. Dietary zinc deficiencies, Zinc-Metallothionein gene interaction, Inflammatory/immune response, Zinc supplementation, Healthy Ageing, Longevity.

Introduction

Aging is accompanied by gradual biochemical and physiological changes including increased susceptibility to diseases and adverse environmental conditions and loss of mobility and agility. The inability of an organism to adjust to the changes may lead to some degenerative age-related diseases, and, to address this, the “remodeling theory of aging” has been proposed [1]. Various nutritional factors can affect age-associated changes. Approximately 40 micronutrients are essential components of the diet. The dietary intake of essential macro- and micronutrients is usually inadequate in the elderly [2] and several factors contribute to this deficiency. Firstly, the poor socioeconomic status of many elderly individuals may lead to a greater consumption of inexpensive foods deficient in micronutrients (e.g., carbohydrates) [3]. Nutrient deficiency is then exacerbated by loss of appetite, lack of teeth, intestinal malabsorption, and decreased energy requirement: all of which can lead to frailty,

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disability, and functional dependence [4]. The deficiency of macro- and micronutrients in elderly is strictly related to global impairment of immune functions, metabolic harmony, antioxidant defense by external noxae and involved in mitochondrial decay [5]. Recent longitudinal studies of daily dietary intake in human centenarians (successful aging) showed that an adequate consumption of micro- and macronutrients leads to good performances in several immune functions, to metabolic compensation, to preservation of antioxidant activity and mitochondria functionality [6]. Therefore, nutritional factors may play a pivotal role in achieving healthy aging and longevity. Among them, zinc is one of the most relevant nutritional factors in aging because it affects the immune response, metabolic harmony, and antioxidant activity, leading to a healthy status [7]. On the cellular level, zinc is essential for proliferation and differentiation and involved in signal transduction and apoptosis [8]. Therefore, it is not surprising that most of the zinc of the human body is located within the cells that make use of a complex homeostatic regulation involving many proteins with a functional role as carriers, buffer and transporters. The distribution of zinc to the cells of our body is dependent upon the plasma pool, which represent the “internal” resource of zinc, that has an average concentration of 12-16 μM and it represents less than 1% of the total body content. Hence, alteration in zinc uptake, retention or secretion can quickly lead to zinc deficiency and affect zinc dependent functions in virtually all tissues and systems, including the immune system [9-11]. Despite its important function, the body has only limited zinc stores that are easily depleted and can not compensate longer periods of zinc deficiency. Additionally, pro-inflammatory cytokines mediate changes in hepatic zinc homeostasis during infections, leading to sequestration of zinc into liver cells and subsequently to hypozincemia [12].

Taking into account that zinc homeostasis is mostly regulated by Metallothioneins (MT) [13] and zinc transporter proteins [14], the interrelationship between zinc and its homeostatic mechanisms is crucial to modulate the inflammatory/immune response in aging in order to prevent disabilities due to age-related diseases. In this study, we review the possible causes of zinc deficiency in ageing, the role of zinc and MT on inflammatory/immune response in aging and in successful aging with a focus and the possible effect of zinc supplementation upon the immune system in old mice and in elderly. A particular emphasis will be given to the effects of zinc supplementation in elderly individuals carrying specific genetic variants of MT and interleukin (IL)-6 genes which, in turn, affect intracellular zinc homeostasis [9].

1. Causes of Zinc Deficiencies

Zinc deficiency is an important factor in the origin of certain common diseases that affect and cause morbidity among the elderly. Zinc is a critical trace element in human health for tissue growth, taste acuity, connective tissue growth and maintenance, immune response, prostaglandin production, bone mineralization, proper thyroid function, blood clotting, cognitive functions, foetal growth and sperm production [15]. Zinc is also required for the biological activity of enzymes, for cell proliferation and for “zinc finger” DNA motifs [16]. Clinical evidences support the pathological consequences that can occur in ageing during zinc deficiency (Table 1). Therefore, the zinc deficiency is a serious public health problem from young up to old age [17]. Such a deficiency in ageing is typically the result of an inadequate zinc dietary intake that may occur as a response to reduced energy requirements or age-related sensory

impairment [18]. It has been reported that mild zinc deficiency is a significant clinical problem in free-living elderly people: only 42.9% have a sufficient intake of zinc (defined as > 67% of the RDA) [19]. These data have been confirmed by other studies (Zenith project [20]; Zincage project [21]) Japan study [22] and German study from the Max Rubner Institute [23]. In the latter, 44% of men and 27% of women (age range 65–80 yrs.) do not reach the recommendation. Moreover, the Third National Health and Nutrition Examination Survey (NHANES) documented a decrease in zinc intake with age, and only 42.5% of participants aged ≥71 years showed an adequate zinc intake (defined as ≥ 77% of the RDA) [24].

Such a reduced zinc dietary intake in ageing seems to induce reduced plasma zinc levels and intracellular zinc ion availability measured using specific fluorescent zinc probe [25]. It is important to note that, despite a reduced intake of zinc, in some old subjects both the plasma zinc levels and the intracellular zinc ion availability may be in the normal range. This outcome suggests that the determination of zinc deficiency is still an unsolved challenge, especially in elderly subjects where chronic inflammatory processes or undiagnosed diseases might complicate the interpretation of the measurements [9]. Anyway, the occurrence of a reduced dietary zinc intake in elderly seems a well documented phenomenon (Zincage project) [21]. Such a reduction may be due to many factors related to the ageing process. Among them, inadequate mastication, psychosocial factors, drug interactions as well as competition between zinc and other bivalent minerals (copper, iron, calcium) may be involved (see chapter 3).

Table 1. Summary of some clinical consequences associated with zinc deficiency in elderly ^a.

Clinical Consequences
Increased total cholesterol
Macular degeneration
Impairment of the immune response
Osteoporosis
Diarrhea
Pneumonia
Possible contributor to loss of appetite
Hypertension and increased risk factor of atherosclerosis
Decreased cell proliferation and increased apoptotic death
Decreased taste acuity
Reduced concentration of transport proteins in the blood
High susceptibility to oxidative damage from certain tissues (brain)
Decreased absorption of dietary folate
Decreased cardiac antioxidant capacity
Mood disorders
Defective connective tissue
Weight loss
Mental lethargy
Decreased resistance to infection, causing an imbalance between Th1/ Th2 paradigm
Abnormalities in cytokine secretion and function
Neuropsychological impairment
Exacerbate hypertension
Decreased functionality in monocytes, natural killer cells, granulocytes and phagocytosis
Risk factor for the development of type 2 diabetes

^a For references see n. [7, 9, 10, 11, 15, 17, 19, 54, 65, 111, 114, 126]

1.1. Mastication and Changes in Oral Structures

Poor dentition and loss of dental pieces are common events among the elderly, while the satisfactory prosthetic replacement option decreases. The inability to masticate properly leads to several modifications in dietary models, since there is a tendency to avoid and substitute certain foods often zinc-rich, such as red meat or hard cheese, with other more soft zinc-poor foods, such as bread. These losses may be also generally caused by periodontal disease, which might in turn be caused by deficits of some minerals in the diet (calcium, phosphorous and zinc) [26]. Although few oral diseases are characteristic in the elderly, some pathological states are frequent in old individuals as compared to young ones, representing a critical factor for a correct zinc intake. The most common oral diseases in old people are especially those that are related to mucose membrane inflammation and/or atrophy, xerostomia (dry mouth), leukoplakia and malignant neoplasia [27]. On the other hand, old subjects with mucosal erythema, stomatitis, angular cheilitis and atrophic glossitis, display low plasma zinc concentrations [28]. Therefore, an improved mastication through individual prosthetic replacement is strongly recommended in elderly in order to increase dietary zinc consumption and, at the same time, to reduce oral mucosa inflammation through an improved mastication.

1.2. Psychosocial Factors

Although the energy requirement is diminished in ageing owing to low physical activity [29] with subsequent higher incidence of diet-related illnesses [30], some psychosocial factors can have a strong impact on dietary habits and consequent low zinc dietary intake. Marital status, depression, mental status, education, socioeconomic status and convenience seem to play an important role in the modification of dietary habits with aging. All these psychosocial factors are also associated with the “frailty syndrome” in old people [31], giving further support to the relevance of the interrelationship among correct diet, psychosocial factors and physical activity for healthy ageing [32]. On the other hand, increased score of depression as well as impaired cognitive performances have been associated with reduced serum zinc levels in a cohort of elderly people [33].

1.3. Drug Interactions

There are numerous cases of adverse drug/nutrition interactions in the elderly. While several drugs (i.e. cefuroxime, erythromycin ethylsuccinate, HMGCoA-reductase inhibitor lovastatin) should be taken with foods to maximize their absorption and efficacy, other drugs (ampicillin, ciprofloxacin, doxycycline, captopril) should not be taken with food in order to have an optimal absorption and efficacy of the drugs [34]. However, drug therapy may frequently interfere with digestion, absorption, utilisation or excretion of essential nutrients altering enzyme biosynthesis, coenzyme or protein transport and hormones metabolism. Moreover, medication can produce appetite, olfactory and taste abnormalities, which in turn affect the nutritional status [35]. Elderly patients usually have more than one permanent daily medication treatments leading to a high risk of interaction between drugs and zinc absorption [36]. One of the mechanisms by which some drugs may interfere with zinc absorption is due to the presence of oxidized metallothioneins that, acting as antioxidant agents to protect the

cells against drug toxicity, provoke a limited zinc capture by enterocytes and no storage of zinc in specific cellular organelles named zinkosomes [37]. As a consequence, the absorption of intestinal zinc is strongly limited and the majority of zinc ion is excreted by urine and the zinc signals, indispensable for cell function, are quenched [38]. Therefore, old people under prolonged drug treatments may need certain nutrient supplements, including zinc, in order to reduce the risk of these interactions. The need of zinc supplementation may also occur by an excessive alcohol consumption being another contributing factor in reducing zinc absorption and subsequent zinc depletion [39] and older individuals are strongly susceptible to the negative effects of excessive alcohol intake [40].

1.4. Cellular Absorption of zinc

Absorption can be considered as the processes of influx into the enterocyte and through the basolateral membrane and of transport into the portal circulation. The subcellular mechanisms involve zinc transporters, MT and a transmembrane transporter DMT1.

Two recently identified families of zinc transporter proteins, ZnT (SLC30) and ZIP (SLC39), have been shown to be responsible for maintaining cellular zinc homeostasis (see chapter 8). The prevailing view of zinc transporter functionality is that transporters in the ZnT family function to reduce cytosolic zinc concentration, either by efflux across the plasma membrane or by intracellular sequestration in subcellular compartments [41]. ZIP family transporters function acts in the opposite direction, to increase cytosolic zinc concentration [42]. Effects of ageing on zinc homeostasis and dietary requirements mediated through effects on zinc transporters are likely to be through transporters with a particular, direct role in intestinal absorption and/or endogenous secretion. In view of their expression patterns and cellular localisation in the intestinal mucosa, members of both families are thought to play an important regulatory role in these processes. Current evidence indicates that, within the ZnT family of transporters, ZnT1, ZnT5 and ZnT6 may be of particular importance in intestinal zinc transport processes. The localisation of ZnT1 to the basolateral membrane of the intestinal enterocyte in rat and mouse [43,44], coupled with evidence that it functions to reduce cytosolic zinc concentration [45], indicates a role for this protein in the efflux of zinc absorbed from the intestinal lumen across the basolateral enterocyte membrane.

The same task also occurs for ZnT5 and ZnT6 that act also in the zinc uptake, other than efflux direction. Indeed, ZnT5 and ZnT6 are responsive to zinc supplementation playing thus a relevant role also for zinc uptake across the enterocyte apical membrane [46]. The localisation and functional and regulatory properties of ZIP4 and ZIP5 indicate that, within the ZIP family, these members play a particular role in the absorption of dietary zinc. ZIP4 is expressed at the apical enterocyte membrane in mouse at increased levels in animals fed a zinc-deficient compared with a zinc-replete diet [47], consistent with a role in dietary zinc absorption. Mutations in ZIP4 are associated with the human zinc deficiency disease, acrodermatitis enteropathica, which displays a strong reduced zinc uptake [47,48]. ZIP5 is believed to play a role in zinc homeostasis through regulated endogenous secretion, by mediating the transport of zinc from the serosa into the enterocyte. This view is supported by the expression of ZIP5 at the basolateral membrane in the zinc-replete, but not zinc-deficient, mouse small intestine [49,50]. It is currently unknown how ageing affects the function, expression or gene regulatory responses to zinc of zinc transporters. However,

a double-blind, randomised, crossover trial examining zinc transporter responses in the small intestine to a daily zinc supplement of 25 mg given orally over 14 days [46], revealed a possible effect of age, but does not allow to make a clear deduction concerning the effect of ageing on gene regulatory responses to zinc.

The role of MT, in the regulation of zinc absorption, particularly in conjunction with the zinc transporters, has been also studied. Hepatic and intestinal MT synthesis is stimulated by dietary zinc supplementation, by intraperitoneal zinc injection and by inflammation and the acute phase response. Dietary zinc restriction also results in diminished MT synthesis. In experiments with knockout and transgenic mice, the rise in serum zinc after a single dose of zinc was much greater in the MT knockouts than in the control animals. In contrast, the serum zinc response of the MT transgenic animals was blunted compared with that of the control animals. The expression of ZnT-1 was also measured and found to be directly related to serum zinc levels but unaffected by MT levels [51]. Thus, MT may work also in intestinal absorption of dietary zinc other than in intracellular buffering of free zinc concentrations [52]. A study compared the effects of changes in zinc status due to zinc supplementation on fractional zinc absorption of young and elderly Korean women [53]. This study suggested that MT individual response adjusted the efficiency of zinc absorption. Indeed, supplemental zinc might increase the synthesis of MT in the intestine, which in turn blocks further absorption of zinc. If the ability to synthesize MT in response to zinc status declines with age [54], the ability to regulate the amount of zinc absorbed may also decline in the elderly. Age may affect MT metabolism by altering the regulation of gene expression, zinc binding ability, or turnover rate [54]. At present, the knowledge around zinc transport mechanisms suggest that also alteration of zinc transporters might be involved in their mechanisms. Further study will be needed in order to detect if the ability to synthesize all the proteins involved in zinc absorption might be affected by the aging process.

1.5. Zinc and Other Mineral Interactions

The interactions between zinc and other minerals are relevant for studying zinc absorption. Iron and calcium are of interest because, although the long-term use of calcium supplements has no effect on zinc status, the calcium content in the diet may, however, affect zinc absorption from phytate-containing meals [55]. Situations that seem most likely to encounter problematic interactions are those in which iron is administered in solution or as a separate supplement rather than being incorporated into a meal [56].

The antagonisms reported in supplementation studies were attributed to a competition between iron and zinc for transport by the divalent metal transporter-1 (DMT1) found in enterocytes of the small intestine [57]. Experiments conducted in Caco-2 cells in presence or absence of fetal bovine serum in the incubating medium have shown that the apical uptake of $^{65}\text{Zn}^{2+}$ was significantly reduced in the presence of iron and serum, suggesting that Fe interferes with the absorption of Zn. The absorption of $^{55}\text{Fe}^{2+}$ was also decreased by excess iron, both in presence and absence of serum. Only in absence of serum, however, the reduction in Fe absorption correlated with a decrease in DMT1 expression [58]. This study implies that Zn uptake may be independent of the DMT1 mechanism. Other mechanisms are involved. A family of human intestinal Zn transporters (ZIP family) was recently identified, suggesting the existence of separate mechanisms for iron and zinc absorption.

With regard to calcium, experiments in animals have shown that calcium interferes in zinc absorption at the intestinal level with a mechanism involving the same transporters on the membrane surface [59,60]. However, in humans, conflicting data exist regarding to the adverse effect of calcium supplementation on zinc homeostasis [61,62]. Therefore, the zinc and calcium interaction and its effect on both metals require further studies.

Zinc also interferes with other micronutrients, such as copper, which homeostasis is similarly regulated by MT. Copper entering the cell can displaces zinc from MT because of the higher affinity of this protein for Cu thus modulating negatively the expression of zinc importers and, consequently, decreasing zinc absorption [63].

2. Zinc Status of the Elderly

The recommended daily allowance (RDA) for zinc in young-adult individuals and older (special recommendations for elderly do not exist) in the United States is 11 mg/day for men and 8 mg/day for women [64]. An uptake below the RDA can only be seen as an indicator of potential zinc deficiency, because many other factors also play a role and the possibility exists that the metabolism may adapt to decreased zinc intake. Hence, it is necessary to analyze the zinc status of each individual. The parameter of choice is often serum or plasma zinc. However, this is not the ideal parameter to determine the zinc status taking into account that many old individuals, despite increased pro-inflammatory cytokines known as factors for zinc depletion [65], display circulating plasma zinc levels within the normal range (about 66-117 µg/dl) [66]. Other parameters may be useful to test the zinc status, such as for example the measurement of total intracellular zinc or intracellular zinc ion bioavailability with specific zinc probes as well as the capacity to release zinc by intracellular MT using NO-donors [67]. Recently, in the Zincage project the determination of the zinc score (based on the determination of the zinc content in the foods and the individual quantity of the food intake) has been shown to represent a possible valid test for the zinc status being well correlated with the age-dependent plasma zinc levels [68]. However, a lot of studies have reported that in general plasma zinc levels decrease with advancing age [69] as well as in some cell types, such as erythrocytes and lymphocytes [19,20]. These findings have been confirmed by data from the second National Health and Nutrition Examination Survey (NHANES), in which serum zinc levels increased into the third decade of life, and declined from that age [70]. Although the upper limit of the dietary zinc intake has not to exceed 25-40 mg/day [71,72], both the dietary zinc intake and the subsequent zinc status decrease with advancing age in various European countries. The European Nutrition and Health Report summarize data regarding the nutritional zinc uptake in elderly from Austria, Denmark, Germany, Hungary, and the UK. Zinc supply decreases with age, although it can be generally regarded as sufficient with however a considerable variation between countries, and zinc uptake is particularly low in UK elderly [73]. Recently, it has been reported from the Zincage project that also in Italy and France the zinc dietary intake in old people may be sufficient whereas in Greece is lower than that one recommended with increased inflammatory markers in comparison with France, Italy and Germany [21].

Studies in a large number of old individuals from various European countries have confirmed this trend with enough zinc dietary intake. Dietary zinc intake and zinc status were measured in a total of 387 old subjects of different age from four different

populations [Clermont-Ferrand (F), Coleraine (UK), Grenoble (F) and Rome (I)][20]. A low predominance of zinc deficiency in free-living and healthy individuals was found in the middle-aged (55-77 yrs.) and over 77 yrs. Daily zinc intake was 11.97 mg/day in males and 10.05 mg/day in females (age range 55-77 yrs.) and 12.04 mg/day and 10.53 mg/day for males and females over 77 yrs., respectively. A Germany study in elderly people (in three different age groups: 65-74, 75-84 and 85 over) living in private households showed that the zinc dietary intake was of 13.06 mg/day in males and 12.19 mg/day in females (age range 65-74 yrs.). In the 75-84 yr. age range, the zinc dietary intake was 12.54 mg/day and 11.53 mg/day for males and females, respectively; in the over 85 yrs., the daily intake was 12.48 mg/day for males and 12.06 mg/day for females [74].

In contrast, a study performed in old institutionalised individuals (60 males and 64 females, age range 65-98 yrs.) in León (E), showed dietary zinc intake lower than that one recommended by RDA independently by the gender (9.4 mg/day for males and 9.1 mg/day in females) [75].

All these findings suggest in general that old people of both sexes display an heterogeneous zinc status and non-optimal zinc dietary intake coupled with marginal zinc deficiency should be addressed individually and within the individual environmental context. First of all, the different dietary habits in different European countries may have a peculiar role. In this context, the Mediterranean diet usually used in Italy, France and in some extent also in Spain, might justify a sufficient dietary zinc intake due to large consumption of food with low-medium content of zinc (cereals, bread, fish, cheese) than consumption of “zinc rich” foods (i.e. red meat). The capability of the human organism to modulate zinc absorption and homeostasis in response to dietary intake might be pivotal to determine the health status in advanced aging. In this context it is remarkable to note that subgroups of elderly subjects can achieve “successful ageing” (centenarian subjects) without suffering from age-related diseases despite a low zinc dietary intake and relatively low levels of plasma zinc [66]. The reason may be related to a better control of the inflammatory status and preserved performance of proteins involved in regulation of zinc homeostasis, such as MT. In these exceptional individuals, the available quota of free zinc ions, despite reduced, in centenarians is still sufficient to maintain good performances in immune response and antioxidant activity because of a lower inflammatory status [76], furtherly confirming the relevance of zinc for a correct inflammatory/immune response.

3. Zinc-Metallothionein (MT) Gene Interaction in Inflammatory/Immune Response and Ageing

Metallothioneins (MT), are a group of low-molecular-weight metal-binding proteins that have high affinity for zinc ($k_d=1.4 \times 10^{-13} M$) [77]. MT exist in different isoforms and sub-isoforms characterized by the length and composition of the aminoacid chain: isoform I, II, III and IV mapped on chromosome 16 in man and on chromosome 8 in mice and display several polymorphic sites [78]. The more common isoforms are I and II; the isoform III is a brain-specific member and the isoform IV is restricted in squamous epithelia. MT contain 20 cysteines, and bind seven zinc atoms through mercaptide bonds that have the spectroscopy characteristics of metal thiolate clusters [77]. MT regulate the distribution of Zn trough direct interaction with other proteins or trough intermediate release of free zinc ions that subsequently bind other proteins [77].

The redox properties of MT are crucial for the protective role of MT in presence of ionizing and UV radiations, heavy toxic metals (mercury, cadmium), lipid peroxidation, reactive oxygen species (ROS), oxidative stress caused by anticancer drugs, and conditions of hyperoxia [79]. This protective role of MT has been especially studied in young-adult MT knockout mice (null mice) for short periods of exposure to toxic metals or in presence of zinc excess or zinc deficiency[80]. Therefore, the protective role of MT is evident in transient stress condition, as it may occur in young adult-age, in which the chronic status (by stress or inflammation) is a rare event [9]. In contrast, this role may be questionable in ageing due to the increased inflammatory and stress-like conditions that might induce irreversible modifications of the Cys residues as shown in the case of homocysteinylation [81] or suspected as in the case of sulfonylation [82]. In the presence of these conditions, these proteins may turn off from protective to harmful agents in ageing following the “Antagonistic Pleiotropy Theory of Ageing” [83]. Increased free zinc in presence of high MT and reduced release of zinc in subjects carrying unfavorable gene variants of MT1a for successful aging [84] and the dose-dependent increase of oxidative stress markers and free zinc in aortic endothelial cells treated with L-homocysteine [85] is in line with this hypothesis.

Therefore, the regulation of the inflammatory response might be the key to understand these mechanisms in successful aging. Since IL-6 acts through its sub-unit receptor gp130, the relative lower gene expression of gp130 in centenarians [76] may imply that a quota of IL-6 is inactive in very old age. As a result, the satisfactory immune performances, metabolic harmony and antioxidant activity may allow a preserved function of MT and a relatively good health status in centenarians [86]. On the other hand, healthy centenarians display preserved MT expression and satisfactory zinc ion availability [86], suggesting the existence of compensatory phenomena able to counteract the effects of inflammation in these exceptional individuals.

Therefore, the role played by the zinc-MT interaction is pivotal to reach successful ageing and, at the same time, to escape some age-related diseases. Since, the persistence of inflammatory stimuli over time represents the biological background favouring the susceptibility to age-related diseases/disabilities, the absence of specific “robust” gene variants and/or the presence of specific “frail” gene variants might predict, on one hand the longevity, on the other hand the predisposition to the appearance of the more common age-related diseases, such as infections and cardiovascular diseases. In this context, it has to be considered that genes selected, because they confer a reproductive advantage early in life, may have dangerous effects in the post-reproductive period. In fact, negative selection against these harmful effects fails due to the decline of natural selection with age. This fact allows that one gene “favourable” in young/adult age may be “disadvantageous” in ageing (antagonistic pleiotropy theory of ageing [83]), as reported above.

Following this perspective, the beneficial effect of inflammation, via an optimal zinc-MT gene interaction, in young and adult age may become detrimental in old age. The recent discovery of novel polymorphisms of MT2A and MT1A supports this assumption. Indeed, old subjects carrying AA genotype for MT2A polymorphism display low zinc ion bioavailability and chronic inflammation by high IL-6, with subsequent elevated risk for atherosclerosis and diabetes type II [9]. By contrast, polymorphism corresponding to A/C (Asparagin/Threonin) transition at position +647 nt position in the MT1A coding region is the most involved in the longevity [84].

Another challenge that remains to be faced is related to the emerging role of extracellular MT in regulating immune response and brain repair mechanisms. MT in

the extracellular environment of the peripheral immune system may support the movement of leukocytes to the site of inflammation representing a "danger signal" for the immune cells and modifying the character of the immune response when cells sense cellular stress. However, a dysregulated production of MT, as may occur during chronic inflammation, may alter the normal chemotactic responses that regulate leukocyte trafficking [87], thus affecting the physiological immune response. The presence of atrophic thymus in young stressed mice overexpressing MT [88] confirm that dysregulated production of MT might affect immune response. However, the real nature of these proteins in aging remains to be clearly established taking also into account that MT may play different role in different organs. Recent findings in cardiac-specific MT transgenic mice suggest that the expression of MT in cardiocytes may alleviate aging-induced cardiac contractile defects and oxidative stress prolonging the life span [89]. In addition, Daf-2 mutant nematodes, other than a longevity phenotype, display an increased expression of MT which, in turn, seem to interact with the insulin signaling pathway[90]. Therefore, even if the specific function of MT in ageing is still a matter of discussion, these last reports, associated to recent findings on the possible role played by MT in modulating energy metabolism [91], strongly suggest that MT is pivotal for maintaining the healthy status [92].

4. Rationale for Zinc Supplementation in Ageing: "in Vitro" Studies

Since the crude zinc balance is negative in old mice and human [92], zinc supplementations in old mice and in elderly have been carried out in order to restore the immune efficiency. The scientific rationale for "in vivo" zinc supplementation finds consistent support by "in vitro" data in immune cells. When PBMCs are stimulated with zinc, IL-1, IL-6, TNF- α , soluble (s)IL-2 receptor and IFN- γ are released [93]. However, the effect of zinc on monocytes may depend by external stimulation. In fact, zinc inhibits LPS-induced TNF- α and IL-1- β release from both primary human monocytes and monocytic cell lines through the inhibition of cyclic nucleotide phosphodiesterase activity [93], suggesting that zinc may also display some anti-inflammatory properties. The dose of zinc used is also a critical variable. In serum-free culture medium, concentrations \geq 100 μ M of zinc stimulate monocytes, but prevent T cell activation, perhaps owing to a lower intracellular zinc content in T cells than in monocytes [93]. Recently, it has been shown that zinc suppresses generation of NF- κ B-regulated inflammatory cytokines by induction of A20 protein in HL-60, human umbilical vein endothelial cells, and SW480 cell lines [94] furtherly supporting a zinc anti-inflammatory role.

Treatment with "in vitro" zinc generally displays different effects on cell survival depending upon the cell type and the dose of zinc used. It seems that both apoptosis prevention and induction are mediated by pathways involving zinc and/or zinc-dependent enzymes [8]. Therefore, the modulation of the intracellular zinc homeostasis plays a key role not only in preventing apoptosis, when oxidative stress or chronic inflammation are low, but also in inducing apoptosis especially when oxidative stress and cellular damage is high in order to down-regulate the immune responses and to eliminate virally infected or malignant cells. Induction of apoptosis by zinc signaling in damaged cells, via activation of p53 pathway, is evident in young-adult age and in very old age [95], perhaps owing to a preserved regulation of zinc homeostasis in both classes of age [86].

Experiments in thymocytes also support this point of view, since media supplemented with zinc from 50 up to 150 µM prevents old thymocyte apoptosis induced by dexamethasone or serum deprivation [96], whereas the direct introduction of free zinc, as zinc-pyrithione, inside thymocytes provokes apoptosis because inducing permanent oxidative stress and irreversible damage [97], thus activating pro-apoptotic pathways. Therefore, zinc supplementation, not exceeding the physiological dose, may be of benefit in ageing either in preventing apoptosis of undamaged immune cells or in reducing the inflammation with a possible prevention of the appearance of age-related diseases.

5. Effect of Zinc Supplementation upon Inflammatory/Immune Response in Ageing

5.1. Old Mice

Old literature reports that a physiological zinc supplementation in the diet throughout the life span in adult rodents prevents some age-related cell-mediated immune modifications [98]. A physiological zinc supplementation (18 µg/ml of Zn²⁺ in the drinking water for 1 month) in old mice induces thymus re-growth and its endocrine activity coupled with an improvement of peripheral NK cell cytotoxicity [92,99]. Zinc supplementation (300mg/kg for 25 days) in aged mice improved thymopoiesis, as assessed by increased total thymocyte numbers [100].

The improved thymic output was mediated in part by reducing the age-related accumulation of immature CD4(-)CD8(-)CD44(+)CD25(-) thymocytes, as well as by decreasing the expression of stem cell factor, a thymosuppressive cytokine [100]. Moreover, old mice treated with daily zinc in drinking water from the pre-senescent age (12-14th month of age) display a significant increment of the mean lifespan when compared to controls [92]. The increased mean lifespan is largely due to significant decrements of deaths for cancer and infectious diseases in the period comprised between 24 and 25 month of age [92]. Of interest, nude and neonatal thymectomized (nTx) mice, which display negative crude zinc balance and a very short life due to thymus absence, show also increased rate of survival after zinc supplementation [54]. Taking into account that the liver extrathymic T-cell pathway is prominent in old, nude and nTx mice in order to compensate the thymic failure [101], it is thus evident the zinc also affects the liver extrathymic T-cell pathway with subsequent good peripheral immune performances, especially liver NKT cells bearing TCRγδ [102].

5.2. Elderly

With regard to elderly, inconsistent data exist on the beneficial effect of zinc supplementation upon the immune efficiency due to different doses and duration of zinc treatment. Although zinc was used at the dose recommended by RDA (from 10 to 25 mg/day with different time length of treatment) (Table 2) in the majority of the studies, Prasad et al. [19] and Boukaniba et al. [103] have found an increment of thymulin activity and improvements in response to skin-test antigens and taste acuity; Bodgen et al. [104] have reported some benefit exclusively for increased lymphocyte mitogen proliferative response; Cakman et al. [105] found enhanced IFN-γ production by leukocytes; Fortes et al. [106] report an increased number of cytotoxic T

lymphocytes; Hodkinson et al.[107] describe no effect on some markers of immunity (NK cells) or inflammation (CRP) but only increased ratio CD4/CD8 T lymphocytes at month 6; Kahmann et al. [108,109] report reduced levels of activated T cells and basal IL-6 release from PBMCs and improved T cell response. Using higher doses of elemental zinc from 40 to 220mg/day with different time length of treatment (Table 1), Duchateau et al. [110] and Sandstaed et al.[111] have observed an improvement in response to skin-test antigens and taste acuity; an improved delayed type hypersensitivity (DTH) reaction has been also found in a limited number of subjects by Cossack [112] and by Wagner et al.[113]; Prasad et al. [114] found improved IL-2 mRNA. Recently, a decrease of inflammatory mediators (CRP, IL-6, VCAM-1) was observed [115]. Other studies [116-118] report instead no effects upon various immune functions after zinc supplementation due perhaps to high dose of zinc used for a too long period (Table 2).

From all these studies, a physiological dose of zinc applied for a long period or high doses of zinc for short periods might induce limited effects on the immune response perhaps due to a zinc accumulation in various organs and tissues with subsequent toxic effect of zinc upon the immune functions [119]. In this context, it is also useful to remind that high doses of zinc trigger apoptosis of the immune cells in presence of high oxidative stress and inflammation [8]. Therefore, caution is advised for the management of zinc supplementation with the suggestion to perform the trial for short periods and on alternate cycles only. In our experience, zinc treatment (15mg Zn²⁺/day for 1 month) in Down's syndrome subjects, in elderly and in old infected patients restores thymic endocrine activity, lymphocyte mitogen proliferative response, CD4(+) cell number, NK cell cytotoxicity and DNA repair as well as thyroid hormones turnover [66,120-122]. At clinical level, significant reductions of relapsing infection occur in Down's syndrome subjects, in elderly and in old infected patients (66,122).

An intriguing point is the increment of zinc transporters after zinc supplementation. Elderly women treated with 22mg of zinc gluconate/day for 27 days display significant increments of ZnT1 gene expression in peripheral leukocytes [123] even if the gene expression of the zinc transporters is sensitive in relation to the immune cells considered [124]. Such increments of ZnT1 have been also observed in human lymphoblastoid cells adding *in vitro* 50 or 100 µmol/L of zinc [123] furtherly suggesting the relevance of zinc supplementation in affecting the gene expression of zinc transporters and, consequently, the correct maintenance of intracellular zinc homeostasis. Such a mechanism might be important for restoring ZnT8 gene expression in pancreatic vesicles being involved in the aetiology of type 2 diabetes [125], pathology related to ageing, to zinc deficiency and altered immune response [126].

Since zinc affects also the cytotoxicity of liver NKT cells bearing TCRγδ (extrathymic T cell pathway) with higher production of IFN-γ in old mice [102], the presence of satisfactory zinc ion bioavailability coupled with increased NKT cell cytotoxicity and enhanced IFN-γ production in centenarians with respect to elderly [127] strengthens the pivotal role of zinc supplementation in maintaining or improving the global immune response (thymic and extrathymic T cell pathways) and in fighting oxidative stress and inflammation. Some authors have also shown that zinc supplementation in combination with other micronutrients may enhance immunity without interfering on vitamin metabolism. Zinc supplementation (dose = 15 or 30 mg/day as zinc gluconate for 6 months) has no deleterious effects on folate or vitamin B12 status in healthy free-living old subjects (age range 55-85 yrs.) [128].

Table 2. Zinc supplementation studies in elderly and old mice: effect upon the immune functions

	Subjects	Number	Intervention^b	Effect
Elderly	institutionalized > 70 y. [ref.110]	15(C) 15(Z)	100mg Zn sulphate one month	increased T cell numbers, DTH, and response to tetanus vaccine
	anergic to DTH, 64-76 y. [ref.113]	5(Z)	55mg Zn sulphate four weeks	improved DTH
	free-living, 60-89 y. [ref. 116]	36(P),36(Z,15) 31(Z,100)	15 or 100 mg Zn acetate, 3 months	no effect on DTH or <i>in vitro</i> lymphocyte proliferation
	zinc-def. males, 65-78 y. [ref. 112]	8(Z)	60mg Zn acetate 4.5 months	increased DTH
	free-living, 60-89 y. [ref. 104]	24(P),20(Z,15) 19(Z,100)	15 or 100 mg Zn acetate; 12 months	negative effect on DTH and NK cell activation after 3 months
	institutionalized, 73-106y. [ref.103]	44(P)/(Z) crossover	20mg zinc gluconate 8 weeks	increased thymulin activity
	zinc deficient 50-80 y. [ref.114]	13(Z)	30mg zinc gluconate 6 months	increased thymulin activity, IL-1, DTH
	institutionalized, 64-100y.[ref.117-118]	190(C) 160(Z)	90mg zinc sulphate 60 days or 30mg 6 months	no effect on antibody response after influenza vaccination and no effect in taste acuity
	institutionalized, ≥ 65y. [ref.106]	30(P) 28(Z)	25mg zinc sulphate 3 months	increased CD4+DR+ T cells and cytotoxic T cells.
	free-living, 65-82 y. [ref.109]	19(Z)	10mg zinc aspartate 7 weeks	reduced activated T helper cells and IL-6 release from PBMC,
	Institutionalized 65-85 y. [ref. 114]	25(P), 24(Z) 6(P), 6(Z)	45mg as gluconate 6-12 months	reduced infections, increased IL-2 mRNA in response to PHA
	healthy, 55-70 y.[ref. 107]	31(P) 28/34(Z)	15/30mg Zn gluconate 6 months	no effect on NK cells or CRP increased ratio CD4/CD8
	healthy, 56-83 y.[ref.115]	20(P) 20(Z)	45 mg zinc gluconate; 6 months	Decreased inflammatory cytokines
	healthy elderly and elderly with COPD (65-85 y.)[ref.66]	15 healthy(Z) 10 COPD(Z)	12 mg zinc sulphate 1 month	Increased NK cell cytotoxicity and active thymulin
	healthy 60-84 y. zinc (\leq 10.5 μ M) [ref. 130]	110(Z)	10 mg zinc aspartate 7 weeks	Increased NK cell cytotoxicity
Old Mice	male Balb/c [ref. 92]	10 (C) 10 (Z)	18 μ g zinc sulphate (22 mg/L in drinking water)	Functional-thymus re-growth; increased NK cytotox.; reduced deaths by cancer, infection
	C57Bl/6 [ref. 99]	12 (C) 12 (Z)	117mg/Kg of zinc in the food vs. 39mg/Kg(C)	Increased thymic lymphocytes; increased thymulin levels.
	Old Balb/c, TX, nude mice [ref. 92]	50 (C) 50 (Z)	18 μ g zinc sulphate	Increased mean survival
	C57Bl/6 [ref. 100]	8 (C); 8 (Z)	300 mg/kg for 25 days	Improved thymic output

^b The values are given as elemental zinc. DTH: delayed type hypersensitivity reaction, (C) control group without supplementation, (P) placebo, (Z) zinc supplementation

However, it is important to highlight that zinc also affects MT gene expression [13]. Therefore, the question arises whether zinc supplementation in old age may further increase MT, with more limited zinc release by MT, as it usually occurs in old age [9]. This fact may be avoided because zinc lowers the inflammation [129] and, as such, MT may be preserved with still a task in zinc release by MT with subsequent good immune performances [84]. Therefore, the potential limited zinc release by MT may be excluded during physiological zinc supplementation in ageing. On the other hand, centenarian subjects, despite the presence of a clear senescent status [67], display lower inflammation and satisfactory zinc status coupled with adequate immune performances and antioxidant activity [66].

5.3. Zinc Supplementation in the Elderly on the Basis of Genetic Background (IL-6 and MT polymorphisms)

One possible cause of the discrepancy existing in the literature on the effect of zinc supplementation upon the immune response in the elderly may be the choice of old subjects who effectively need zinc supplementation in strict relationships with dietary habits and inflammatory status. This assumption is supported by the discovery that old subjects carrying GG genotypes (termed C- carriers) in IL-6 -174G/C locus display increased IL-6 production, low intracellular zinc ion availability, impaired innate immune response coupled with enhanced MT. By contrast, old subjects carrying GC and CC genotypes (termed C+ carriers) in the same IL-6 -174 locus displayed satisfactory intracellular zinc as well as innate immune response [130]. But, the more intriguing finding is that male C+ carriers are more prone to reach centenarian age than C- carriers. Therefore, old C- carriers are likely to benefit more from zinc supplementation than old C+ carriers. The distribution of IL-6 -174 genotype is very different among various European Countries with large differences between Northern and Southern European Countries [21]. Zinc supplementation in old C- subjects restores NK cell cytotoxicity to values present in old C+ carriers and considerably improves both zinc status, assessed by the percentage increment of granulocyte Zn [131] and stress response, assessed by the percentage increment of MT protein as well as Clusterin/Apolipoprotein J and other proteins related to oxidative stress and inflammation [132]. When the genetic variations for IL-6 polymorphism are associated with also the variations of MT1A +647A/C gene, the plasma zinc deficiency and the altered innate immune response is more evident [131], suggesting the genetic variations of IL-6 and MT1A are very useful tools for the identification of old people who effectively need zinc supplementation. These results open the hypothesis that the daily requirement of zinc might be different in elderly harbouring a different genetic background. Such a role played by the genetic background on the beneficial effect of zinc supplementation is also evident in keeping the pro inflammatory cytokine and chemokine productions better under control [131] as well as in reducing the gene expression of genes related to the inflammatory status, such as IL-1 and its receptor [133]. A very intriguing point is also the beneficial effect of zinc supplementation in cognitive performances from old individuals selected on the basis of IL-6 polymorphism [134]. A comprehensive portrait of the effect of zinc supplementation on zinc status, immune response, cytokines, chemokines and stress-related proteins in old people selected on the basis of IL-6 and MT polymorphisms is provided in Table 3.

Table 3. Effect of zinc supplementation in elderly according to genetic background and zinc status^c.

	Parameter	Effects
Zinc Status [ref. 132,133]	Plasma Zinc	↑
	Plasma Zinc/Albumin	↑
	Labile intracellular zinc	↑↑
	Metallothioneins	↑
	NO-induced release of zinc	↑↑
	Granulocyte Zinc	↑↑
	MT glutathionylation	-
Stress-related Proteins [ref. 21, 130,132]	poly(ADP-ribosyl)ation capacity	↑
	ROS production	↓
	ApoJ plasma	-↑
	Genes involved in Nitrosative stress (ATF2, CSF2, FOS, ICAM1, JUN, LTA, CCL2, SELE, VCAM1, iNOS, TNF, NFKB1)	↓
	Total intracellular carbonyl levels	↓
	MsR activity and protein expression	↑↑
	Chymotrypsin-like peptidase activity of proteasome and 20S protein expression	↑
	Chaperone (Hsp72) protein levels	-↑
	Chaperone (Hsp72) inducibility	↑↑
Antioxidant Plasma enzymes [ref. 21, 132]	Plasma SOD	↑
	Erythrocyte SOD	↑
	Catalase	↓
	Glutathione Peroxidase	↓
Thymic output [ref. 21, 132]	T-cell receptor excision circles (TRECs)	↓↑
Senescence and apoptosis [ref. 21, 132]	Telomere length	-↑
	Early Spontaneous Apoptosis	↓
	Late Apoptosis	↓
	Oxidative stress-induced apoptosis	↓
	Mitochondrial membrane depolarization during spontaneous and dRib induced apoptosis	↓
	Cell Cycle	-
Plasma Cytokines and Chemokines [ref. 131]	IL-6, IL-8, MIP-1α	-↑
	MCP-1, RANTES	-
Immune functions [ref.131]	NK lytic activity	↑↑
	Basal IFN-γ, IL-8, IL-1ra and IL-6 production	↓
	Basal IL-10 and TNFα production	↓
	Stimulated IFN-γ, IL-6, TNF-α, IL-1ra and IL-10 production	↑
Jak/Stat signalling and immunomodulation [ref. 21,131,132]	IL-2 and IL-6 STAT3 and STAT5 activation	-
	Activation-induced cell death (AICD)	↑
	Cytokines and metabolic gene expression response to zinc	↑↓
T cells subsets [ref. 21,131]	Activated T cells (CD3+CD25+)	↓
	CD4:CD8	-
	Frequencies of CMV-specific cells	-
Cognitive functions [ref. 134]	Perceived Stress Scale	↓

^c Legend: ↑↑ strongly increased; ↑ increased; - not modified; -↑ slightly increased; ↓↑ intervariability; ↓ decreased

6. Conclusions and Perspectives

Zinc deficiency in elderly, resulting mainly from the reduced zinc dietary intake together with some age-related factors (mastication, psychosocial factors, drugs interactions, cellular processes), could compromise many body homeostatic mechanisms, including the immune functions, leading to the appearance of some age-related degenerative diseases. Since zinc deficiency is a common event in the elderly, several researchers have documented the impact of zinc supplementation in old people in order to restore the zinc status and, as such, to prevent the disability caused by the diseases. Clinical evidences have also suggested that zinc-rich foods, as occurring in the Mediterranean diet, may be useful in the prevention of zinc deficiency in old people, as shown in some European countries (Italy and France).

However, controversial findings exist on the "real" necessity of zinc supplementation because the major problem for zinc supplementation in old people is related to the choice of old subjects who effectively need zinc supplementation. The sole determination of plasma zinc is not sufficient because zinc is bound to many proteins. The intracellular zinc ion availability and the zinc release by MT can be used as complementary methods to test zinc status. The polymorphisms of IL-6 and MT1A may be the added value to screen effective old subjects for zinc supplementation in restoring inflammatory/immune response. As a consequence, the healthy ageing and longevity may be achieved. The prolonged survival observed in old, nude and Tx zinc treated mice and the escaping of infection relapses in old infected patients after zinc supplementation may be in line with this interpretation. However some points require further investigations. First of all, the reason of the limited zinc release in ageing and the biochemical mechanism involved, in particular addressing NO-related intracellular pathways. However, IL-6 and MT1A polymorphisms may form a solid rationale to select old individuals who effectively need zinc supplementation and not the entire old population. Moreover, some aspects of the zinc absorption have to be better studied because it is dependent by the interactions with other nutrients (copper, iron, calcium). Concomitantly, the precise dose of zinc to be used and the length of the treatment have also to be considered. Anyway, zinc and MT gene interaction is crucial for a correct inflammatory/immune response in order to reach healthy ageing and longevity.

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17. Zinc and Atherosclerosis: Clinical Observations and Potential Mechanisms

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Abstract. Zinc is an essential nutrient that is required for a broad range of biological functions. The potential exists for zinc to impact on chronic disease. The aims of this chapter are twofold: firstly to evaluate the epidemiological and clinical data that report on zinc status and atherosclerosis in humans, and secondly to determine the potential mechanisms of the interaction of zinc with atherosclerosis risk factors. There are conflicting reports of the relationship between atherosclerosis and zinc status, as assessed by dietary intake of zinc and/or the measurement of zinc concentrations in healthy and diseased tissues. The balance of epidemiological studies points to an association between zinc deficiency and atherosclerosis; however the studies are hampered by the lack of a decisive biomarker of zinc status. Clinical trials are mainly of zinc supplementation, and these show a decrease in plasma high-density lipoprotein cholesterol concentrations leading to an increased risk of heart disease. Impaired zinc homeostasis has been associated with increased levels of oxidative stress and the induction of widespread genomic and proteomic changes that relate to cardiovascular disease. Potential mechanisms of the influence of zinc on atherogenesis studied in rodent models and in cell culture include its interaction with a wide range of cellular redox and inflammatory processes, such as NF- κ B, NO, PPAR, and PKC signalling pathways. In conclusion, zinc is likely to be involved in atherogenesis through its interactions with lipoprotein metabolism, inflammation and oxidative stress. Further progress will be made when improved methods of measuring zinc status are developed.

Keywords. Zinc status; atherosclerosis; lipoprotein metabolism; inflammation; oxidative stress

Introduction

Zinc is necessary for a wide range of physiological processes. In addition to its numerous structural and catalytic functions, zinc is involved in the regulation of an extensive variety of genes, impacting such diverse processes as protein-protein interactions, fatty acid metabolism, apoptosis, and signal transduction [1,2]. Thus the potential exists for zinc to influence many metabolic functions and to impact a range of diseases.

The aim of this chapter is to examine the relationship between zinc status in humans and the development and progression of atherosclerosis. Reports of epidemiological and clinical studies that measure zinc concentrations in populations

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with cardiovascular disease will be evaluated. Potential mechanisms underlying the role of zinc in atherosclerosis will be explored, with particular emphasis on plasma lipoprotein metabolism, inflammation and oxidative stress-related risk factors.

1. Zinc Homeostasis

The adult human contains approximately 2 – 3 g of total body zinc, which has a ubiquitous and variable tissue distribution. As there is no recognised storage site for zinc, cells are dependent on plasma to supply them with sufficient zinc to sustain normal function. In healthy persons, plasma zinc concentrations are maintained within a narrow range (approximately 10 to 18 µmol/L).

Synergistic adaptations in zinc absorption and excretion in the gastrointestinal tract are the primary means of maintaining a constant state of total body zinc. At the cellular level, an intricate arrangement of homeostatic mechanisms has evolved to regulate the intracellular zinc content and its distribution within the mammalian cell [3]. Although much is yet unknown, the maintenance of cellular zinc homeostasis is believed to involve complex interactions between zinc sensors, such as metal responsive element-binding transcription factor-1 (MTF-1), and cell signalling machinery; the transcriptional and/or post-translational regulation of the ZnT (SLC30) and Zip (SLC39) transporter families; the trafficking of zinc through the cell by metallothionein (MT); and the turnover of proteins that bind zinc with high affinity (see chapters 4 and 8).

2. Zinc Deficiency

Zinc deficiency was first described in 1961 with clinical features of stunting, infantile genitalia, enlargement of the liver and spleen (hepato-splenomegaly), and accumulation of fluid (oedema). Among the biochemical features, plasma total cholesterol concentrations were reported to be in the range of 105-245 mg/dL (2.7-6.3 mmol/L) [4]. Other features of severe zinc deficiency include infections of the epithelium and poor wound healing, diarrhoea, and decreased appetite.

Although severe zinc deficiency is relatively rare in developed countries, less acute deficiency states are believed to be highly prevalent (see chapter 3). The determination of mild to moderate zinc deficiency is made difficult by the lack of a sensitive and specific biochemical marker of zinc status. The most obvious factor attributed to the development of zinc deficiency is an inadequate intake of dietary zinc, often in conjunction with high intakes of phytic acid, a potent inhibitor of zinc absorption. Deficiencies associated with low intakes of absorbable zinc are exacerbated during times of increased requirement, including growth, pregnancy, and lactation. In addition to diet-induced zinc deficiency, pathologic conditions may cause or contribute to a zinc deficient state. Gastrointestinal disorders can result in reduced intestinal absorption and/or reabsorption of zinc. Conditions such as chronic diarrhoea, excessive burns, or traumatic and surgical wounds increase endogenous zinc losses, while numerous chronic diseases, including atherosclerosis, have been associated with impaired zinc utilisation.

3. Zinc Status and Atherogenesis

Ischaemic or coronary heart disease (CHD) is the most common form of heart disease and a significant cause of premature death. CHD is almost always due to atherosclerosis, which is a chronic, inflammatory disorder of complex aetiology. Atherosclerosis is characterised by the infiltration of lipids into the arterial intima that are taken up by macrophages to form foam cells, and culminates in the formation of fibrous plaque. Plaques may rupture, thus allowing blood to enter and disrupt the arterial wall, and this often leads to thrombosis and vascular spasms. Coronary arteries are particularly susceptible and so atherosclerosis is the major underlying pathology of coronary artery disease (CAD). Clinical manifestations of atherosclerosis include myocardial infarction (MI), heart failure, angina and sudden death. Cerebral and peripheral arteries can also be affected by atheroma and collectively cardiovascular disease (CVD) encompasses diseases of the heart and the peripheral arteries.

Epidemiological studies suggest that the progression of atherosclerosis is modified by lifestyle and nutritional factors [5-7]. While much of the research focus has been on macronutrients, zinc status also appears to be an important modifier of the atherosclerotic process, with research reports indicating both a contribution of zinc deficiency to its development and a protective effect of zinc in the disease.

3.1. *Population Studies of Zinc Status*

A number of studies of varied design have investigated zinc status in atherosclerosis or its complications (Table 1). In the absence of a reliable biomarker, serum or plasma zinc and dietary zinc intake are the most widely used indicators of zinc status, although a range of other variables, including urinary, hair, toenail, and various tissue levels of zinc, also have been investigated.

3.1.1. *Plasma or Serum Zinc and Atherosclerosis*

Large ecological studies describe an inverse association between serum zinc and CVD mortality in the population generally [8-10] as well as in cohorts at substantial risk of CVD events [11,12]. In a cross-sectional study of patients undergoing routine coronary angiogram, lower serum zinc was observed in patients with angiographically-defined CAD compared to those with normal angiogram [13].

Low serum or plasma zinc has been reported in patients with CHD [14, 15,16], carotid artery stenosis [17], low-grade carotid atherosclerosis [18], ischaemic cardiomyopathy [19], and ischemic stroke [20]. Lower serum zinc has been observed in CAD patients compared to dyslipidaemic patients without CAD and healthy controls [21]. Similarly, serum zinc has been reported to be lower in patients with atherosclerosis obliterans (AO) [22] than in controls.

In contrast, no association between serum zinc and CAD [23-25] or ischaemic cardiomyopathy [26] has been reported. In AO patients, serum zinc has been found to be either unaffected [27,28] or higher [29-31] in those with femoral or iliac AO compared to controls.

When taken together, these studies show that perturbations of zinc homeostasis may occur in either direction; however the balance of evidence suggests that serum or plasma zinc concentrations are decreased in CVD.

Table 1. Zinc Status in Humans with Atherosclerosis or Related CVD Complications

Study (author, year)	Participants	Study Design	Measure of Zn status (Zn in compartment/ tissue)	Outcome
Volkov, 1963 [32]	36 M, 36 F patients with atherosclerosis (deceased); 20 healthy donors, 18 individuals who died as a result of accident as controls	case-control	plasma; erythrocyte; liver, aorta, myocardium, pancreas, adrenal glands, kidneys	Zn was decreased in plasma, aorta, liver, myocardium, & pancreas compared to controls
Chipperfield & Chipperfield, 1973 [33]	19 patients who died from coronary thrombosis or myocardial degeneration; 14 controls	case-control	heart muscle	No significant difference in Zn status
Atsumi & Numano, 1975 [22]	20 patients with AO; 47 controls	case-control	serum	Serum Zn lower in patients with AO compared to controls
Versieck et al., 1975 [34]	16 patients with AMI; 46 controls	case-control	serum	Serum Zn lower in AMI patients compared to controls
Sullivan et al., 1979 [14]	42 patients with arteriosclerotic heart disease; 37 controls	case-control	serum	Low serum Zn in patients compared to controls
Manthey et al., 1981 [23]	106 patients undergoing coronary arteriography	cross-sectional	serum	No association between serum Zn & prevalence & severity of CHD
Speich, 1982 [35]	12 M, 14 F who died from AMI; 15 M, 12 F who died of trauma as controls	case-control	myocardium (left and right ventricles) & aorta; necrotic area of left ventricle (AMI)	Zn was lower in the left ventricle necrotic area of patients who died after AMI than in left ventricle tissue from control subjects; Zn was higher in right ventricle and non-necrotic left ventricle tissue after AMI compared to corresponding control tissues
Khan et al., 1984 [36]	27 patients with myocardial ischaemia but without infarction; 56 AMI patients; 26 patients with previous MI; 50 healthy controls	case-control	serum	Serum Zn was lower in AMI patients; the lower Zn levels in AMI patients were relatively higher in samples collected 72 h after the episode compared to samples taken at 0-10 h

Aalbers & Houtman, 1985 [37]	200 deceased donors; The Netherlands	cross-sectional	heart, liver, kidney, aorta, rib, hair	Inverse correlation between Zn in aorta wall with sclerosis of the aorta; MI patients had lower Zn in liver
Cichocki et al., 1985 [38]	72 yr old M suffering from diabetes & atherosclerosis (3 samples taken from different sites of the popliteal artery)	case study	tunica adventitia, tunica media & tunica intima layers of popliteal artery	Highest mean concentration of Zn was found in the tunica media
Ringdal et al., 1986 [39]	13 M, 8 F MI patients (deceased); 13 M, 9 F controls (deceased)	case-control	kidney, liver, spleen, heart, pancreas, brain	No difference in Zn concentration between the MI & control groups
Tiber et al., 1986 [40]	55 M patients with CAD	cohort	plasma	Plasma Zn was within normal range
Bialkowska., 1987 [41]	29 M survivors of MI; 23 M controls	case-control	hair	Hair Zn higher in survivors of MI
Dubick et al., 1987 [42]	14 patients with atherosclerosis; 29 AA patients; control tissue from 9 deceased adults	case-control	aortic wall	Zn was lower in diseased samples without evidence of calcified plaque compared to those with plaque
Bakos et al., 1988 [43]	23 M, 7 F AMI patients; 7 M, 3 F noncardiac patient controls	case-control	serum	Within 72 h of admission, no difference in serum Zn of AMI patients compared to controls
Kok et al., 1988 [44]	62 CVD deaths & matched controls from the Epidemiological Study of Cardiovascular Risk Indicators; The Netherlands	case-control	serum	CVD deaths did not differ from controls in mean level of serum Zn
Piorunskaa-Stolzmann et al., 1988 [29]	17 M with femoral AO; 12 M with abdominal AA due to atherosclerosis	cohort	serum; arterial wall of lower limb	Higher Zn in serum & arterial wall in patients with AO compared to those with abdominal AA
Mendis, 1989 [45]	71 deceased M accident victims	cross-sectional	aorta	Higher Zn in fibrous plaques compared to healthy tissue
Oster et al., 1989 [15]	27 patients with CHD; 88 healthy controls	case-control	serum; right auricle of heart	serum Zn lower in CHD patients compared to controls
Uza & Vlaicu, 1989 [27]	160 patients with AO; 67 controls	case-control	serum	Mean level of serum Zn did not differ between AO patients & controls

Mikkelsen et al., 1992 [46]	67 patients with CHD; 47 healthy individuals	cross-sectional	plasma	Tendency for lower plasma Zn in patients with CHD
Peltomaa et al., 1992 [47]	19 M, 12 F deceased individuals	cross-sectional	calcified atherosclerotic plaques & plaque-free vessel walls of carotid artery	Upper end of range for Zn in plaque was four times greater than corresponding value in vessel wall
Suciuc et al., 1992 [48]	100 IHD patients; 100 subjects with history of MI; 150 patients with AMI; 50 controls	case-control	serum	Serum Zn values higher in patients with IHD and history of MI & lower in AMI patients compared to controls
Tan et al., 1992 [49]	29 M, 12 F AMI patients; 41 healthy matched controls	case-control	serum	Lower plasma Zn in AMI patients compared to controls
Iskra et al., 1993 [28]	16 M with femoral atherosclerosis; 18 M controls	case-control	serum	No difference in plasma Zn between males with atherosclerosis & controls
Oster et al., 1993 [50]	20 M, 7 F patients with CHD undergoing bypass surgery	cohort	right auricle	No correlation between heart Zn & occurrence of MI or severity of CHD; Zn was correlated with (cardiac) ejection fraction
Vlad et al., 1994 [51]	40 patients who had died of CHD; 16 control individuals who died from other causes	case-control	abdominal aorta	Lower Zn in atherosclerotic plaques of those who died from CHD compared to control tissue
Reunanen et al., 1996 [8]	230 men with CVD; 298 matched controls	case-control	serum	Low serum Zn associated with increased mortality from CVD
Iskra et al., 1997 [30]	27 M with femoral AO; 25 M with AA; 18 M controls for serum Zn only	cohort	serum; aortic wall; plaque	Serum Zn was higher in AO patients compared to AA patients & controls; Arterial Zn higher in AO compared to AA; Zn higher in plaque than surrounding tissue
Martin-Lagos et al., 1997 [19]	12 patients with MI; 9 patients with ischaemic cardiomyopathy; 80 healthy individuals	cross-sectional	serum	Serum Zn lower in patients with ischaemic cardiomyopathy but not different in MI patients compared to healthy individuals

Marniemi et al., 1998 [52]	344 community-living elderly	cohort	serum	Serum Zn not associated with CVD mortality
Singh et al., 1998 [9]	1769 rural, 1806 urban M & F; North India	cross-sectional	dietary intake; serum	Low Zn intake & low serum Zn associated with increased prevalence of CAD & CVD risk factors
Falkiewicz et al., 2000 [53]	30 patients with metabolic syndrome (20 with CHD)	cohort	hair	Lower hair Zn than in other published studies
Iskra & Majewski, 2000 [31]	14 M, 15 F with femoral or iliac AO; 12 healthy M controls	case-control	serum	Higher serum Zn in AO group compared to controls
Mielcarz et al., 2001 [24]	22 patients with angiographically-defined CAD; 44 patients with normal arteries	cross-sectional	serum; leukocyte	No difference in serum or leukocyte Zn in patients with CAD
Nyström-Rosander et al., 2002 [54]	46 patients with aortic stenosis; 15 deceased individuals without known CVD as controls	case-control	aortic valve	Higher Zn in sclerotic compared to control valves
Martin-Moreno et al., 2003 [55]	624 men with a first AMI; 724 controls; European multi-centre case-control study on antioxidants, MI & breast cancer (EURAMIC)	case-control	toenail	Toenail Zn not associated with AMI
Lee et al., 2005 [56]	34492 postmenopausal women from Iowa Women's Health Study	cohort	dietary intake	Zn intake not associated with CVD mortality overall; Zn intake & CVD mortality inversely correlated in those who consumed ≥ 10 g alcohol/d
Alissa et al., 2006 [57]	130 M with established CVD; 130 age-matched controls; Saudi	case-control	serum; urine	No difference in serum Zn between groups; urinary Zn lower in CVD patients compared to controls
Leone et al., 2006 [10]	4035 M from Paris Prospective Study 2	cohort	serum	CVD mortality tended to be inversely associated with serum Zn
Giacconi et al., 2007 [17]	288 patients with carotid artery stenosis; 218 age- & sex-matched healthy controls	case-control	plasma; erythrocytes; PBMC (free Zn)	Less free Zn in PBMC & lower plasma & erythrocyte Zn in patients with stenosis compared to controls
Kazemi-Bajestani et al., 2007 [13]	114 patients undergoing routine coronary angiogram; Iran	cross-sectional	serum	Lower serum Zn in patients with angiographically-defined CAD

Soinio et al., 2007 [11]	1050 patients with type 2 diabetes mellitus; Finland	cohort	serum	Low serum Zn is independent risk factor for CHD
Costarelli et al., 2008 [18]	10 adults with low-grade carotid atherosclerosis; 10 healthy matched controls; Italy	case-control	plasma; PBMC (free Zn & NO-induced Zn release)	Plasma Zn & NO-induced release of Zn were lower & free Zn was higher in patient PBMC compared to controls
Ghayour-Mobarhan et al., 2008 [21]	55 CAD patients; 183 dyslipidaemic patients without CAD; 135 controls	case-control	serum	Lower serum Zn in CAD patients
Kazi et al., 2008 [58]	130 MI patients; 61 healthy age-matched healthy controls; Pakistan	case-control	whole blood; urine; hair	Whole blood & hair Zn lower & urinary Zn higher in MI patients
Kerkeni et al., 2008 [16]	100 CHD patients; 120 healthy controls; Tunisia	case-control	serum	Lower serum Zn in patients with CHD compared to controls
Stadler et al., 2008 [59]	16 deceased donors; The Netherlands	cross-sectional	carotid artery; abdominal aorta	Higher Zn in advanced lesions than in healthy tissue or early lesions
Pilz et al., 2009 [12]	3316 patients from the Ludwigshafen Risk & Cardiovascular Health study	cohort	serum	Serum Zn associated with mortality; adjustments for CVD risk factors attenuated association with CVD mortality to marginal significance
Shokrzadeh et al., 2009 [26]	30 ischaemic cardiomyopathy; 27 controls	case-control	serum	No difference in serum Zn of patients & healthy controls
Afridi et al., 2010 [60]	457 M CVD patients; 536 healthy controls	case-control	whole blood; urine; hair	Whole blood & hair Zn lower and urinary Zn higher in CVD patients compared to healthy subjects
Falcone et al., 2010 [61]	298 HIV-positive adults from cardiovascular substudy (CARE) cohort of Nutrition for Healthy Living study	cross-sectional	serum	No association between serum Zn & carotid IMT
Giannoglou et al., 2010 [25]	72 patients without prior history of MI who underwent coronary angiography	cross-sectional	serum; urine	No association between serum Zn & CAD; urinary Zn loss higher in patients with CAD & positively associated with CAD severity; serum Zn/24 h urine Zn ratio inversely associated with CAD

Munshi et al., 2010 [20]	256 ischemic stroke patients; 180 matched controls	case-control	serum	Lower serum Zn in stroke patients compared to controls
Yang et al., 2010 [62]	4564 adults free of clinical CVD; South Korea	cross-sectional	dietary intake	Zn intake was inversely related to subclinical atherosclerosis

AA: aortic aneurysm; AMI: acute myocardial infarction; AO: atherosclerosis obliterans; CVD: cardiovascular disease; CAD: coronary artery disease; CHD: coronary heart disease; F: female; IMT: intima-media thickness; IHD: ischaemic heart disease; M: male; MI: myocardial infarction; NO: nitric oxide; PBMC: peripheral blood mononuclear cells.

3.1.2. Longitudinal Changes in Serum Zinc

The conflicting nature of observations in studies exploring the relationship between serum zinc and atherosclerosis may be explained by differences in the extent of the disease, the site of atherosclerosis, or confounding factors [63]. Time of measurement of plasma or serum zinc in relation to a CVD event may explain differences in observations in some instances. In cases of acute myocardial infarction (AMI), serum zinc concentrations measured soon after infarct have been shown to be lower in AMI patients compared to patients with myocardial ischaemia without infarction or healthy controls [34,48,49]; however, less of a difference or no difference has been found with measurements taken 72 hours after the episode [36,43]. Longitudinal changes in serum zinc levels after AMI consistently show an immediate and acute decrease in zinc levels after AMI onset that is sustained in the first 48 hours before the zinc concentration gradually returns to initial levels (Table 2).

It has been hypothesised that the fall in serum zinc concentrations in AMI could be due to the large amount of zinc needed by the myocardial tissue to participate in the process of repair after infarction [64]. Zinc variations could be explained also by responses to various forms of stress; the release of leukocyte endogenous mediator (LEM) in response to infection or inflammation has been shown to depress plasma zinc concentrations and increase hepatic zinc uptake [65]. The increase in zinc uptake by tissue could be mediated by changes in the zinc-binding capacities of serum proteins. Of the major protein fractions in serum, zinc is bound principally to albumin, and a relationship between the metabolism of zinc and albumin has been reported [66,67]. A correlation has been shown after AMI between the total concentration of zinc in serum and that of albumin-bound zinc, suggesting that the fraction of zinc bound to serum albumin accounted for most of the changes in the total serum zinc concentration [68].

Table 2. Time Course of Changes in Zinc Status in Humans after Acute Myocardial Infarction

Study (author, year)	Participants	Study Design	Measure of Zn status	Outcome
Wacker et al., 1956 [69]	AMI patient	case study	serum	Decrease in serum Zn after AMI, returning to normal by day 16
Halsted & Smith, 1970 [70]	26 AMI patients; 89 controls	case-control	plasma	Plasma Zn fell within 24-48 h after MI, returning gradually to normal over 2 wk period
Handjani et al., 1974 [71]	18 patients (11 with AMI, 7 with myocardial ischemia without infarction)	Cohort	serum	Serum Zn level fell sharply in AMI patients within a day of onset, returning to normal within 7-10 d; no change from normal values in serum Zn of patients with myocardial ischemia
McBean et al., 1974 [72]	12 M, 1 F with AMI; 5 M with myocardial ischemia; 10 M, 10 F controls	case-control	serum	Serum Zn was low after AMI & returned to normal within 4 to 24 d; no change from normal values in serum Zn of patients with myocardial ischemia
Low & Ikram, 1976 [73]	88 patients with AMI; 48 patients with ischaemic changes; 52 without CVD	cross-sectional	plasma	Plasma Zn fell in AMI patients, returning to normal by day 10; Zn levels in AMI patients lower than group with ischaemic changes & those without CVD
Walker et al., 1978 [74]	14 AMI patients; 16 myocardial ischaemia patients; 30 healthy controls	case-control	plasma; albumin	Plasma Zn fell after AMI, reaching the lowest level at day 2 & returning to normal by day 8; plasma albumin fell progressively in AMI patients, being lowest on day 8
Lewandowicz et al., 1979 [75]	40 AMI patients; 13 controls	case-control	serum	Serum Zn was lower in AMI patients compared to controls; fall in serum Zn significantly correlated with AMI severity
Ponteva et al., 1979 [76]	47 AMI patients; 30 patient & 7 healthy controls	case-control	plasma	Plasma Zn content was significantly lower in AMI patients compared to healthy (but not patient) controls
Lekakis & Kalofoutis, 1980 [77]	99 AMI patients; 40 patients with angina pectoris & chest pain but without infarction	case-control	serum	Low plasma Zn in AMI patients compared to patients with chest pain but without infarction; plasma Zn fell in AMI patients in first 3 d after onset before gradually increasing
Zumkley et al., 1980 [78]	31 patients with AMI; 40 healthy controls	Cohort	plasma	Low plasma Zn in AMI patients, returning to normal by day 7

Parashar & Fernandes, 1982 [79]	50 AMI patients; 27 patients with chest pain but without significant IHD; 23 patients with IHD but without MI	Cohort	plasma	AMI patients exhibited a fall in plasma Zn within the first 3 d that returned to normal by day 10; a correlation was observed between Zn level and severity of arrhythmia	
Khan et al., 1984 [80]	27 patients with myocardial ischaemia; 56 AMI patients; 26 patients with previous MI	case-control	serum	Serum Zn in AMI patients was lower than controls; lower Zn levels in AMI patients were relatively higher in samples collected 72 h after episode compared to those taken at 0-10 h	
Speich et al., 1987 [81]	34 M, 8 F AMI patients; 58 M, 53 F controls	case-control	plasma; erythrocyte	On day 1 of MI onset, plasma Zn was lower in AMI patients & erythrocyte Zn was higher in female AMI patients compared to controls; plasma Zn returned to control levels by day 10	
Speich et al., 1988 [64]	26 M, 11 F with pre-infarction syndrome; 34 M, 8 F who survived AMI; 6 M, 4 F with AMI leading to death; 58 M, 53 F without CVD	cross-sectional	plasma; erythrocyte	Plasma Zn reduced in first 3 d after AMI returning to normal levels in days 6 to 12; in patients with AMI leading to death, plasma Zn lower compared to those without CVD, plasma Zn lower in days before death compared to plasma Zn in patients who survived AMI, no differences in erythrocyte Zn	
Katayama et al., 1990 [82]	44 M, 17 F AMI patients	Cohort	serum Zn	Zn level fell within first 24 h with lowest level on day 2; returned to normal by day 5, continued to increase up to day 10	
Jain & Mohan, 1991 [83]	30 AMI patients	Cohort	serum	Serum Zn declined in the 24 h after MI onset until day 4 & increased to normal by day 14	
Arnaud et al., 1994 [68]	21 AMI patients	Cohort	serum; ligands	Zn-binding	Serum Zn & albumin-bound Zn declined up to day 3 then returned gradually to reference ranges
Pucheu et al., 1995 [84]	18 patients with AMI onset, 16 patients with CAD	case-control	plasma	Decrease in plasma Zn levels after onset of thrombolytic treatment (maximum at 12 h); returned to normal by day 3	
Vilanova et al., 1997 [85]	60 AMI patients; 56 healthy matched controls	case-control	serum	Decrease in serum Zn in AMI, returning to normal at the time of cardiac enzyme normalization; at 48 h after onset, serum Zn was lower in patients who died compared to those who survived	
Gomez et al., 2000 [86]	32 AMI patients; 32 healthy matched controls	case-control	serum; albumin-bound Zn; globulin-bound Zn	Serum Zn lower in AMI patients in 10 d following onset with lowest level at day 3, gradual increase after day 3 to initial values; correlation between total serum Zn & albumin-bound Zn	

AMI: acute myocardial infarction; CAD: coronary artery disease; CVD: cardiovascular disease; F: female; IHD: ischaemic heart disease; M: male; MI: myocardial infarction.

3.1.3. Zinc in Other Biological Compartments

In addition to serum or plasma zinc, the zinc content of a variety of biological compartments has been investigated (Table 1). Studies generally report that urinary zinc concentrations are higher in CVD compared to controls [25,58], indicative of impaired zinc utilisation in the disease. In three observational studies, hair zinc has been reported to be lower in patients with MI [58], CVD [60] and the metabolic syndrome [53]. In contrast, hair zinc was found to be higher in male survivors of MI compared to controls [41]. Toenail zinc was found not to be associated with AMI in a multi-centre case-control study [55].

Zinc levels in whole blood have been found to be lower in MI [58] and CVD [60] patients compared to healthy subjects, while no difference was observed in leukocyte zinc in patients with advanced CAD compared to controls. Zinc concentrations in erythrocytes have been shown to be lower in patients with carotid artery stenosis compared to age- and sex-matched healthy controls [17]. Data on free zinc concentrations in atherosclerosis are conflicting [17,18].

The zinc content of the aorta, liver, myocardium, and pancreas was shown to be lower in patients who died of atherosclerosis compared to individuals who died as a result of trauma [32]. In accord with these observations, a cross-sectional study of 200 deceased individuals in the Netherlands reported an inverse relationship between zinc in the aorta wall and sclerosis of the aorta, and lower hepatic zinc concentrations in those who died from MI compared to individuals with no history of MI [37]. On the other hand, no difference was found between patients who died of MI and controls in the zinc content of the kidney, liver, spleen, heart (with or without infarction), pancreas, or brain [39].

The site of sample extraction may account for inconsistencies in the reported zinc contents of specific tissues. In a case study of a 72-year old male with atherosclerosis, zinc was found to be highest in the tunica media layer of the popliteal artery in comparison to the tunica adventitia and tunica intima layers [38]. In a case-control study, zinc was shown to be lower in the necrotic area of the left ventricle of patients who died after AMI compared to those who died of acute trauma; in the right ventricle and the non-necrotic left ventricle, zinc in samples from AMI patients was higher than that of the corresponding control tissues [35]. Zinc concentrations have been found to be significantly lower in atherosclerotic plaques of the abdominal aorta of patients who died as a result of CHD compared to normal aortic tissue samples [51]. In other studies, higher zinc levels have been reported in atherosclerotic plaques compared to surrounding tissue [30,45], in sclerotic compared to control aortic valves [54], in diseased aortic wall samples with calcified plaque compared to those without evidence of plaque [42], and in advanced lesions of the carotid artery or abdominal aorta compared to healthy tissue or early lesions [59].

Differences in the type and extent of CVD and sites of tissue sampling, as well as the lack of standardised units for the expression of zinc concentrations in different tissue types, complicate comparisons between studies. The consistent theme in the majority of reports, however, is that zinc homeostasis in CVD is perturbed.

3.1.4. Relationship of Atherosclerosis with Dietary Zinc

Few studies have explored the relationship between dietary zinc intake and atherosclerosis (Table 1), despite zinc intake being a common indicator of zinc status.

A recent study investigated the relationship between dietary zinc intake and carotid intima-media thickness, a surrogate marker of subclinical atherosclerosis. It demonstrated in the population overall a higher mean intima-media thickness in those in the lowest quintile of dietary zinc intake compared to those in the highest quintile group. After adjusting for potential confounders, dietary zinc intake was inversely correlated with subclinical carotid atherosclerosis, suggesting a protective role of dietary zinc intake against the development of CVD [62]. A number of factors may influence the relationship between dietary zinc and CVD, including gender and alcohol consumption; in a cohort of nearly 35000 postmenopausal women from the Iowa Women's Health Study, an inverse association between zinc intake and CVD mortality was observed in those women who consumed ≥ 10 g alcohol/d but not in the study population overall [56]. In a cross-sectional study conducted in an Indian population, lower zinc intakes were associated with an increased prevalence of CAD and CVD risk factors; however a significant omission in the study design was the lack of adjustment for dietary factors that appeared to be associated with zinc intake [9] and which are known to affect CVD risk significantly [5-7], making these results difficult to interpret. Further studies are needed to determine the relationship between zinc intake and CVD.

4. Potential Mechanisms

The mechanisms by which zinc may be involved in the development and progression of atherosclerosis and CVD more generally have been widely hypothesised; in particular, the relationship of zinc to lipoprotein metabolism appears to be a key consideration.

4.1. Zinc and Lipoproteins

Dyslipidaemia is a risk factor for atherogenesis. The major changes in lipid profile in CVD are an increase in plasma total cholesterol and triglycerides, a reduction in high density lipoproteins (HDL), and the increased appearance of low density lipoproteins (LDL) (Table 3), especially those particles classified as small, dense LDL, which are particularly susceptible to oxidative modification. LDL particles infiltrate the arterial sub-endothelial space where they may trigger inflammatory processes that ultimately lead to the formation of atherosclerotic plaque [87]. HDL, which is responsible for cholesterol efflux from peripheral tissues, may oppose this process and the reduction of HDL cholesterol in CVD has been associated in numerous studies with increased disease risk [88]. A number of reports suggest that zinc may impact the risk of atherosclerotic disease by influencing lipoprotein metabolism.

Table 3. Characterisation of Plasma Lipoproteins

Lipoprotein	Major constituent		Association with atherosclerosis
	Lipid	Apoprotein	
VLDL ¹	Triglycerides	B100, C, E	Increase risk
LDL ²	Cholesterol, cholesteryl esters	B100	Increase risk
HDL ³	Cholesteryl esters, phospholipids	AI, AII, C, E	Decrease risk

¹ Very low-density lipoprotein; ² Low-density lipoprotein; ³ High-density lipoprotein

4.1.1. Human Studies

4.1.1.1. Zinc Deficiency

Under controlled conditions, depletion of zinc per se was induced in humans by feeding diets that contained 3 mg Zn/d. These initial studies, published in abstract form, report a decrease in plasma total cholesterol of 22% [89], and a decrease in LDL cholesterol [90]. There is insufficient evidence to determine the effect of zinc depletion in humans on cholesterol metabolism. Most studies have focused on the effects of an increased zinc status, through supplementation, on plasma lipoprotein concentrations.

4.1.1.2. Zinc Supplementation

Increasing the exposure of males to zinc through supplementation of 160 mg Zn/d, four times the upper limit of recommended daily zinc intake, resulted in a 25% decrease in serum HDL cholesterol concentrations [91]. Upon cessation of the supplemental zinc, serum HDL returned to baseline within approximately 9 weeks. In young women, daily zinc supplementation using 3 doses of zinc (15, 50, 100 mg) produced a transient decrease in HDL cholesterol concentrations but no clear dose-dependence [92]. In a series of case studies in the elderly [93], a population considered to be at high risk of zinc deficiency, supplementation with high doses of zinc (300 mg/d) confirmed the reported detrimental effect on HDL [91]. These studies additionally describe an increase in LDL cholesterol concentrations with zinc, suggesting a further increase in CVD risk.

A meta-analysis of controlled clinical trials determined the effect of zinc supplementation on plasma lipoprotein cholesterol and triglyceride concentrations in humans [94]. Thirty-three interventions comprising a total of 14,238 subjects were included in the random effects meta-analysis. In the overall analysis, no effects of zinc supplementation were observed for plasma total cholesterol, LDL cholesterol, HDL cholesterol or triglyceride concentrations. Secondary analyses (Figure 1A) revealed that zinc supplementation is associated with a significant decrease in plasma HDL cholesterol concentrations in individuals classified as healthy (n=13,215). Conversely, in smaller numbers of subjects with type 2 diabetes mellitus (DM) (n=151) or those undergoing haemodialysis (n=40), zinc supplementation was associated with an

increase in HDL cholesterol concentrations, suggesting that the effects of supplementation may be dependent on health or underlying zinc status. When compared to baseline values, the mean change in healthy subjects was a 7% decrease in HDL cholesterol concentrations, an amount considered to be clinically significant. In participants with type 2 DM or those undergoing haemodialysis, plasma HDL cholesterol concentrations increased by 34% and 18%, respectively, conferring a significant decrease in CHD risk. The increase in HDL cholesterol in individuals with type 2 DM or undergoing haemodialysis may be mediated by insulin. Insulin has been proposed as an independent predictor of plasma HDL and zinc ions have been shown to influence insulin signalling [95]. The view that zinc may have a beneficial effect in chronic disease is supported by the observation that a higher plasma zinc concentration protects those with type 2 DM from cardiovascular complications [11]. In the meta-analysis [94], the increase in plasma zinc after zinc supplementation was significantly higher in those with type 2 DM than in healthy subjects, despite these two groups having similar plasma zinc concentrations at baseline, suggesting that an underlying defect in zinc homeostasis may have a significant effect on lipoprotein metabolism.

Further secondary analyses demonstrated that the influence of zinc on cholesterol levels exhibit a dose-response [94]; interventions that administered higher doses of elemental zinc (≥ 100 mg/d) were associated with a significant decrease in total cholesterol levels (Figure 1B), which appears to be explained at least in part by the decrease in HDL. It has been hypothesised that one potential mechanism of action by which high intakes of zinc impact cholesterol metabolism relates to the induction of copper deficiency [96]. In humans, zinc supplementation decreases biomarkers of copper status [97, 98].

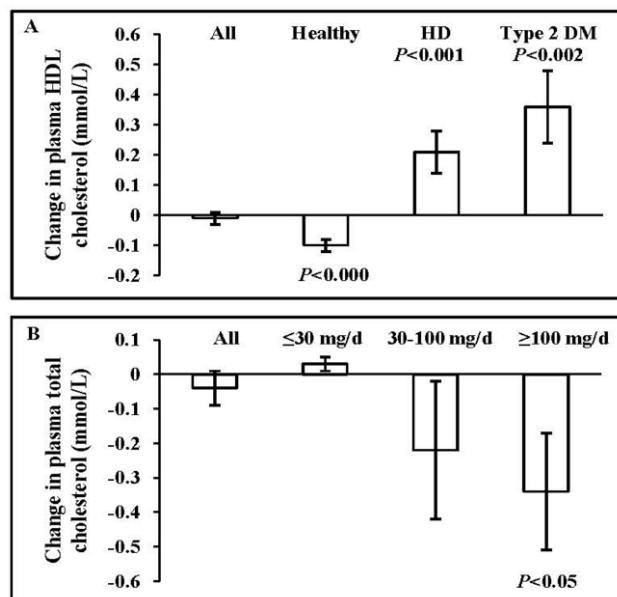


Figure 1. [A] Effect of Zinc Supplementation (shown as elemental zinc) on Plasma HDL Cholesterol Concentrations by Health Status: All (n=14,122), Healthy Individuals (n=13,215), Haemodialysis Patients (n=40), Type 2 DM (n=151); [B] Effect of Elemental Zinc Dose on Plasma Total Cholesterol Concentrations.

4.1.2. Studies in Animal Models and In Vitro

Although the mechanisms by which zinc exerts an effect on cholesterol metabolism are unknown, in animal and human cell-line experiments zinc deficiency is associated with significantly reduced levels of HDL and a concomitant reduction in plasma apolipoprotein A-I levels [102,103]. In rats, zinc deficiency decreases the efficiency of cholesterol absorption [104] and enhances bile acid excretion [105]. On the other hand, zinc supplementation has been shown to decrease the extent of incorporation of radiolabelled methionine in HDL suggesting a decrease in HDL synthesis [106].

Some reports suggest that zinc is needed for the activity of lecithin:cholesterol acyltransferase (LCAT), which circulates in plasma bound to HDL and serves to increase its cholesteryl ester content. In animal studies, it has been reported that LCAT activity is lowest in zinc deficient rats and that cholesterol esterification increased with increases in dietary zinc [107]. In vitro experiments of the effects of zinc on LCAT in human plasma have demonstrated both an inhibitory [108] and a stimulatory [109] effect of the metal on the enzyme activity. Although the potential mechanism of an effect of zinc on LCAT activity remains to be clarified, it has been shown that the regulation of the human LCAT promoter involves Sp1 [110], a transcription factor that contains the classic Cys₂His₂ zinc finger motif and which utilises a 'redox zinc switch' in its regulatory function [111].

4.2. LDL Oxidation

Atherosclerosis is characterised by increased levels of oxidised lipids in the vessel wall [112] and it is postulated that the oxidation of lipoproteins is an important early event in the pathogenesis of the disease. Oxidised LDL (oxLDL) has been shown to play a critical role in abnormal endothelial vasorelaxation [113] and uptake of oxLDL can disrupt the endothelium, injuring endothelial cells or committing them to apoptosis. In macrophages of the atherosclerotic plaque, oxLDL has been demonstrated to elevate levels of particular matrix metalloprotein isoforms [114], a group of zinc-containing endopeptidases that have important roles in the metabolism of extracellular matrix and which appear to be critically involved in vascular remodelling.

4.2.1. Human Studies

Few studies have explored the effects of zinc on LDL oxidation in humans. In healthy young men supplemented with 50 mg Zn/d, no significant changes were observed in the propensity for LDL to undergo oxidative modification [115], and supplementation with 15 or 30 mg Zn/d had no effect on in vitro LDL oxidation parameters in healthy elderly subjects [116]. In a randomised controlled zinc intervention trial in prepubescent children with metabolic syndrome, supplementation with 20 mg Zn/d was demonstrated to significantly decrease levels of oxLDL, C-reactive protein (CRP), and malondialdehyde [99]. The effects in adults of varying zinc doses on oxLDL concentrations in disease states are unknown.

4.2.2. Studies in Animal Models and In Vitro

In vitro, zinc has been shown to inhibit the oxidation of LDL by cells or transition metals in a concentration-dependent manner [117]. Further, zinc deficiency has been demonstrated to interfere with peroxisome proliferators-activated receptor (PPAR)

signalling [118,119]; PPAR γ , which is highly expressed in foam cells in atherosclerotic lesions, can be activated by oxLDL constituents and appears to possess an antiatherosclerotic effect [120].

Although the potential for changes in cellular zinc homeostasis to modulate oxLDL levels in atherogenesis is unclear, the complex relationship between zinc and oxidative processes more generally is emerging as an important field of inquiry in chronic disease research.

4.3. Zinc, Oxidative Stress, and Inflammation

Reactive species (RS), including reactive oxygen and reactive nitrogen oxide species, have important physiological roles in a wide range of signalling pathways, but their accumulation can place cells in a state of oxidative stress. Increased oxidative stress has been related to many key atherogenic events, including endothelial damage or dysfunction, modulation of phosphorylation signalling, and alterations of nitric oxide (NO) availability; disturbances in redox homeostasis have been linked to chronic inflammation via such mechanisms as the induction of proinflammatory cytokines (including IL-1, IL-6, and TNF- α), and the aberrant activation of NF- κ B, PPAR, and toll-like receptor (TLR) signalling pathways. Each of these redox-sensitive processes appears also to be influenced by intracellular zinc concentrations.

Zinc, despite being redox-inert and therefore not itself an antioxidant, exhibits a variety of indirect antioxidant effects (see chapter 4). The interaction of zinc with cell membranes stabilises them against damage [121]. Zinc enhances the antioxidant capacity of the cell through direct competition with metals that are known to catalyse the Fenton reaction, such as copper and iron. It is integral to the activity of superoxide dismutase (SOD) and is able to induce the synthesis of MT and glutathione, all of which protect against an accrual of reactive species in cellular systems. Further, zinc modulates the functions and protein-protein interactions of numerous redox-responsive proteins at several levels of signalling cascades [111], and may itself act as a signalling ion [122] (see chapter 6). The ease with which labile zinc is transported into endothelial cells [123] suggests that the vascular endothelium may be particularly influenced by perturbations in zinc homeostasis and metabolism [124].

4.3.1. Human Studies

A study comparing the zinc status and MT expression of peripheral blood mononuclear cells isolated from healthy and atherosclerotic patients showed that the intracellular distribution of zinc is altered by atherosclerosis [125]. In a randomised controlled placebo trial in 40 ostensibly healthy elderly subjects, supplementation with 45 mg Zn/d for 6 months was associated with an increase in antioxidant power and a decrease in plasma concentrations of CRP, IL-6, macrophage chemo-attractant protein 1 (MCP-1), vascular endothelial cell adhesion molecule 1 (VCAM-1), and oxidative stress markers, indicating that zinc has an atheroprotective effect in this population [126]. As proposed in type 2 DM and in patients undergoing haemodialysis [94], it is conceivable that zinc supplementation addressed an underlying perturbation in zinc homeostasis, given that elderly populations are considered to be susceptible to mild to moderate zinc deficiency [127] (see chapter 6).

The idea that populations believed to be zinc deficient are more likely than healthy individuals to benefit from zinc supplementation is supported by multinutrient

supplementation studies investigating CVD outcomes. On the one hand, a large primary cardiovascular and cancer prevention study of healthy participants, which incorporated 20 mg of zinc as part of its antioxidant supplementation protocol, showed no effect of supplementation in the prevention of CVD [128] or on carotid atherosclerosis and arterial stiffness [129]. In a randomised nutrition intervention trial in 29,584 participants selected from the general population of Linxian, China, treatment groups receiving a combination of zinc, vitamin A, riboflavin, and thiamine demonstrated a lower incidence of stroke mortality for high-risk subjects (age ≥ 60 y, systolic blood pressure ≥ 160) but not for others [130]. Further trials are needed to determine the antioxidant effects of supplementing zinc alone in individuals exhibiting CVD risk factors.

4.3.2. Genomic and Proteomic Studies

Studies by Cousins et al. [131] and Beattie et al. [132] indicate that zinc depletion induces widespread genomic and proteomic changes that relate to CVD and redox signalling. Genes identified by global microarray screening as dysregulated in a zinc deficient human monocytic leukemic cell line (THP-1) were found on analysis to fit a pattern that is analogous to defective macrophage activation in intact animals [131]. In rodent models of acute and marginal zinc deficiency, proteomic analysis ascertained changes in structural, carbohydrate, and fatty acid-related protein clusters in the aorta that appear disadvantageous for maintaining vascular health; the structural effects are suggestive of changes in smooth muscle contractility and the deficiency-induced suppression of carbohydrate and fatty acid metabolism is indicative of insulin resistance and perturbation of key zinc-dependent transcription factors, such as the PPAR proteins and classical PKC isoforms [132].

Increased cellular zinc concentrations also have been shown to have global effects on gene and protein expression. Kindermann et al. [133] performed DNA array and proteome analysis in a human colonic epithelial cell line (HT-29) after the cells were exposed to a zinc concentration that increased intracellular free zinc but did not cause toxicity. Most of the molecular targets that responded with changes in steady state expression levels to increased intracellular zinc could be linked to an impaired state of cellular ATP production and the cellular stress response.

4.3.3. Studies in Animal Models and In Vitro

In vitro and in vivo animal studies support a cardioprotective effect of zinc that often involves an interaction with redox signalling pathways or a reduction in oxidative stress. Zinc has been shown to reduce catecholamine-induced cardiac oxidative injury [134,135] and preserve post-ischemic function in models of cardiac ischemic injury [136,137]. Hearts from rats receiving dietary supplementation of the zinc ionophore pyrithione, recovered fully from ischemia/reperfusion, via a mechanism that was reported to involve the zinc-mediated protection from degradation of PKC isoforms [138]. Zinc supplementation of mice with chemically-induced diabetes was found to protect against diabetic cardiomyopathy, an effect that purportedly was mediated by cardiac MT induction [139].

Intracellular zinc concentrations have been linked to NF- κ B signalling [140]. NF- κ B is ubiquitously expressed and impacts an extensive assortment of cellular processes, including proliferation, immunity, inflammation, and apoptosis. It is a key component of the adhesion molecule upregulation process; is involved in the promotion of smooth

muscle cell proliferation [141]; and mediates signal transduction by toll-like receptors (TLR), which play an important role in the initiation of the innate immune response and are implicated in the development and progression of atherosclerotic disease [142]. Cellular zinc deficiency has been shown to upregulate NF- κ B activity in endothelial cells [119,143] and high levels of NF- κ B have been found to be present in the smooth muscle cells of the atherosclerotic lesion [144]. These observations suggest a role for zinc in the early stages of atherogenesis through interactions with NF- κ B.

The release of NO by the endothelium plays a key role in vascular homeostasis. A release of intracellular zinc from proteins containing zinc-sulphur complexes, stimulated by inducible nitric oxide synthase (NOS)-derived NO, has been shown to be a critical component of an Nrf2-dependent signalling pathway that activates the GSH redox cycle in endothelial cells, ultimately protecting against oxidative damage [145]. The zinc-dependent enzymes CuZnSOD and extracellular-SOD function to protect the cellular availability of NO by controlling superoxide levels, which if unchecked can lead through the formation of peroxynitrite to endothelial NOS uncoupling, itself an important mechanism of pathologic superoxide production in the vascular endothelium [146]. The ability of zinc to protect the redox-signalling functions of NO is likely to be ameliorated by a zinc deficient state or perturbed zinc utilisation in atherosclerosis.

Zinc interacts with a wide range of cellular redox and inflammatory processes, including NF- κ B, NO, PPAR, and PKC signalling pathways. Impaired zinc homeostasis, and in particular zinc deficiency, has been associated with increased levels of oxidative stress and the induction of widespread genomic and proteomic changes that relate to CVD.

5. Conclusion and Perspectives

Knowledge of the multitude of biological pathways affected by zinc in health and disease continues to expand. There are numerous indications in human, animal, and *in vitro* studies that zinc exhibits many functions that affect heart disease [63,94,111,124]. Localised zinc deficiency or the potential for zinc to be redistributed aberrantly among target proteins and intracellular compartments during the atherosclerotic process likely ameliorates this protective effect and there are a number of positive indications for zinc supplementation in CVD. The desirability of zinc supplementation needs to take account, however, of the potential for adverse effects; supplementation with high zinc doses is recognised to produce adverse consequences similar to those observed in zinc deficient conditions, such as the inhibition of T-cell functions and aberrant expression of cytokines [147]. Such results indicate the need for caution in the administration of zinc in the clinical setting.

Further well-designed randomised controlled trials are necessary to provide cogent insight into safe and desirable levels of zinc supplementation in atherosclerosis and to determine the extent to which the results of *in vitro* and animal studies are relevant to human health. It appears likely that zinc deficient subjects will react differently to zinc supplementation than zinc sufficient ones; however interpretation of results is made difficult by the lack of a sensitive and specific biomarker of zinc status. Further investigation of the molecular mechanisms that underpin the transport, sensing, and distribution of zinc is required to further our understanding of zinc homeostasis and ability to determine zinc status in humans.

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18. Zinc Homeostasis and Signaling in the Brain

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Abstract. The role of zinc in the brain was a subject of intense interest, based particularly on the observation that a chelatable zinc pool is found at glutamatergic synaptic vesicles and is co-released during synaptic activity. Studies utilizing nutritional zinc deficiency or chelation linked brain zinc to cell death during ischemia and a role in development as well as learning and memory. Numerous studies in recent years led to identification of multiple zinc transporters among them the ZnT3 that is responsible for zinc accumulation in the vesicles. Generation of transgenic animals lacking specific zinc transporters together with the availability of zinc dyes led a dramatic progress in our insight on the role of brain zinc homeostasis. In this chapter we will discuss mechanisms by which zinc induces neuronal signaling or neuronal death, and how these may link zinc to learning and memory, seizure, ischemia and Alzheimer's disease (AD). Finally, we will focus on the emerging importance of zinc signaling in glia and glial-neuronal interaction.

Keywords. Neuron; Chelatable zinc; ZnT3; ZnR, ischemia; seizure.

Introduction

A free zinc pool, termed chelatable zinc, is mostly found in glutamatergic vesicles throughout the brain and upon its staining a remarkable picture emerges. This unique distribution that was already discovered in the fifties was followed by yet another important finding, that this pool of zinc is released during synaptic activity. The importance of synaptic zinc is underlined in numerous studies describing developmental brain abnormalities and enhanced susceptibility to seizure that are associated with nutritional zinc deficiency or zinc chelation. In contrast, brain zinc was shown to enhance cell death during ischemia and seizure. A series of studies on the interaction of synaptic zinc with postsynaptic receptors identified multiple allosteric zinc binding sites of varying affinities on the NMDA, Glycine and GABA receptors. Studies employing ischemia and seizure models have further suggested that zinc rise in postsynaptic neurons induces neuronal death. Yet other studies suggested that the chelation of the synaptic zinc pool leads to neuronal death, indicating that the role of zinc is far more complex. In monitoring brain zinc transport early studies employed Ca^{2+} sensitive dyes that bind Zn^{2+} at much higher affinity. Nevertheless the vast access

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of Ca^{2+} over Zn^{2+} often reduced the sensitivity of these measurements. Another major obstacle was that the molecular identity of the Zn^{2+} transporters was unknown and hence knowledge on brain Zn^{2+} homeostasis was incomplete. In the last 20 years specific zinc homeostatic and signaling machinery was identified. Thus, the ZnT and ZIP families of zinc transporters were discovered as well as activity of a $\text{Na}^+/\text{Zn}^{2+}$ exchanger and a distinct Zn^{2+} sensing receptor (ZnR). The role of these transporters in neurons and glia cells has begun to shed light on the mechanisms of zinc in physiology. This progress was complemented by generation of zinc dyes with exceptional affinity and selectivity that can detect minute changes in zinc concentrations. In this chapter we will describe the field following these leaps and the challenges that lie ahead.

1. Zinc Transport in the Brain

The importance of the Zn^{2+} homeostatic machinery is highlighted by the fact that the number of Zn^{2+} homeostatic proteins is larger than the number of proteins linked to Ca^{2+} transport [1]. Zn^{2+} homeostasis is particularly dynamic in the brain and was shown to play a crucial role in the survival of neurons and glial cells [2].

1.1. ZnT Family

Members of the zinc transporters (ZnT, solute carrier 30) family, except for ZnT1, are found on many types of intracellular organelles underscoring the importance of controlling Zn^{2+} content in each of these compartments. It is well accepted that the ZnT transporters are responsible for lowering cytoplasmic Zn^{2+} . Expression of ZnT proteins is also tightly regulated by Zn^{2+} itself and a rise in cellular Zn^{2+} content enhances their expression [3-6]. Fluorescent analysis and functional modeling have recently led to the elucidation of the mechanism of the ZnTs and the identity of their Zn^{2+} binding site [7]. These studies that used ZnT5 show that expression of this ZnT member is linked to accelerated removal of cytoplasmic Zn^{2+} and enhanced uptake of vesicular Zn^{2+} [8]. They further demonstrate that vesicular Zn^{2+} uptake is linked to accelerated alkalinization of the Golgi apparatus, indicating that ZnTs are acting as $\text{H}^+/\text{Zn}^{2+}$ exchangers. Finally, molecular modeling of ZnTs had identified the ZnT binding site that consists of 4 residues, 2 Asp and 2 His, which coordinate the transport of Zn^{2+} ions. It was also shown that ZnTs are forming homo- and hetero- dimers and that this oligomerization is important for their targeting and activity. For example, the heterodimerization of ZnT5 and ZnT6 is not critical for the catalytic activity of ZnT5, but is essential for Zn^{2+} delivery into the Golgi apparatus, which is required for the folding and stability of the tissue non-specific alkaline phosphatase enzyme, TNAP, which is a Zn^{2+} -dependent enzyme [9, 10]. Similarly the homo-oligomerization of ZnT3 leads to targeting of this protein into the synaptic vesicles [11]. It is still not clear how the hetero-oligomerization enhances the transport of Zn^{2+} . However, studies of the bacterial ZnT homologue Yiip indicate that residues at the dimeric interphase of this transporter are critical for Zn^{2+} transport [12]. Intriguingly residues at the same region of ZnT are associated with enhanced susceptibility to type-2 diabetes [13, 14]. ZnT3 can also functionally interact with synaptic transporters. For example, ZnT3 is co-localized with the vesicular glutamate transporter 1 (Vglut1) and zinc transport mediated by ZnT3 also accelerates glutamate transport, likewise glutamate transport enhances zinc transport mediated by ZnT3 [15].

In the brain, ZnT3 is arguably the most extensively studied ZnT member. It is mainly localized on the synaptic vesicles in glutamatergic boutons [16, 17]. Recent studies however suggest that the expression of ZnT3 is not limited to the glutamatergic neurons and is also found at the GABAergic neurons in the brain and spinal cord, where it also promotes synaptic zinc accumulation [18, 19]. The importance of Zn²⁺ in learning and memory (see chapter 19) and brain development considered together with the profound effects of its deficiency on these functions led to the hypothesis that synaptic Zn²⁺ is essential for brain function. Despite the potential importance of ZnT3, early studies employing the ZnT3 knockout (KO) mice were seemingly disappointing considering the mild phenotype observed in these mice [20, 21]. The synaptic transmission seemed normal in the ZnT3 KO mice and learning and memory functions that were tested were not significantly impaired. These mice did show enhanced susceptibility to kainate induced seizures highlighting the importance of synaptic Zn²⁺ in enhancing neuronal inhibition [21, 22]. Since the synaptic Zn²⁺ pool is not the only pool found in the brain, this model proved to be extremely useful as it enabled, for the first time, to specifically dissect and analyze the role of synaptic zinc in a wide range of physiological and pathophysiological processes, ranging from neuronal excitability to the etiology of Alzheimer's disease (AD) (see chapter 21) and brain ischemia (see chapter 22) [23]. Indeed, an important study utilizing the ZnT3 KO mice assessed neuronal death in the hippocampus following seizure, and showed that it was much more extensive in the KO mice than in WT mice that have a rich synaptic Zn²⁺ pool in this region [24]. Thus it was suggested that the "toxic Zn²⁺" pool is the intracellular pool and not the synaptically stored Zn²⁺. Consistent with this observation, numerous works indicate that the rise in intracellular Zn²⁺ is enhanced by nitrosative and oxidative stress that trigger the release of this ion from intracellular Zn²⁺ pools, most prominent is its release from metallothioneins [25-29]. ZnT3 and the role of the synaptic Zn²⁺ pool have been also studied in the context of Alzheimer's disease [23]. Gender difference in the size of plaques disappeared in the ZnT3 KO mice. Furthermore, in female mice estrogen appeared to decrease the expression of ZnT3, and thereby of synaptic Zn²⁺, by altering the expression of ZnT3 adaptor protein complex AP-3 [30]. The link between ZnT3 and AD was further shown in a study showing that the expression of ZnT3 as well as the content of Zn²⁺ in the brain drops dramatically in AD patients [31]. Most important however, while young ZnT3 KO mice show normal cognitive function they exhibit a marked age dependent deficit in learning and memory fully manifested at 6 months [31]. Remarkably, these deficits are associated with alteration of key hippocampal proteins linked to learning and memory among them most notable AMPA receptors, NMDA receptor subtypes 2a and 2b and elements of the brain-derived neurotrophic factor (BDNF) pathway such as pro-BDNF and tyrosine kinase receptor, trkB. Similarly, apolipoprotein E ablation linked to AD leads to the reduction in expression of ZnT3 and AP-3, which is required for the synaptic targeting of ZnT3 [32]. Thus, ZnT3 by facilitating the uptake of synaptic Zn²⁺ appears to be important for age dependent preservation of the synaptic machinery and critical for learning and memory. Further studies on the role of synaptic Zn²⁺ in cognitive function focused on the amygdala, which is another prominent source of synaptic Zn²⁺. These have shown that ZnT3 KO mice are deficient in associative fear memory and extinction [33].

ZnT1 is linked to resistance to Zn²⁺ toxicity and protects neurons and glia cells from cytoplasmic Zn²⁺ rise associated with excitotoxic and physical insults [3, 4, 34]. Expression of ZnT1 in brain slices confers protection against ischemia or seizure

induced brain damage. Furthermore there is a strong developmental link between appearance of synaptic Zn²⁺ and the expression of ZnT1 in the CA3 region of the hippocampus [35]. Although it is unclear if the expression of ZnT1 is triggered by the synaptic Zn²⁺ or a rise of Zn²⁺ in the soma of the neurons in these regions. In cerebral ischemia the induction of ZnT1 expression is triggered by a rise in cellular Zn²⁺, induced by nitric oxide, indicating that this pool of Zn²⁺ is more dominant in triggering the expression of ZnT1 [29]. Although the protective role of ZnT1 is well established, its mode of action is still unclear. Expression of ZnT1 was suggested to enhance Zn²⁺ efflux [4]. In agreement, ZnT1 has the catalytic core of 2 His and 2 Asp required for coordination of Zn²⁺ transport via H⁺/Zn²⁺ exchange. The H⁺ gradient however across the plasma membrane is not steep enough to promote the formation of the transmembrane Zn²⁺ gradients observed in cells. Other studies indeed indicated that ZnT1 is reducing cation influx mediated by the L-type Ca²⁺ channels (LTCC) presumably by interacting with these channels [36].

Other members of the ZnT family are also found in the brain and there are indications for changes in their expression and distribution during pathphysiological processes. For example, in the vicinity of AD plaques, expression of several ZnT proteins was shown to be elevated, among them ZnT5 and ZnT6 in the periphery of the plaques while ZnT7 was observed in the core of the plaques [37]. Similarly, changes in many of these transporters was observed following cerebral ischemia [29]. Further work is however required to elucidate their physiological role in the brain.

1.2. Zn²⁺ Exchange Mechanism

The steep Zn²⁺ gradient that is found in the neurons may reach 4-5 orders of magnitude particularly during synaptic activity. Such gradients may need to be maintained by the activity of pumps or secondary active Zn²⁺ transporters. Heavy metal pumps that can also transport Zn²⁺ are found in bacteria and plants [38] but there is no evidence for such pumps in mammalian cells. The presence of a Na⁺ dependent Zn²⁺ exchanger has been documented in several studies [39-41]. Although the molecular identity of this exchanger has not been elucidated it shares a functional similarity with the mammalian Na⁺/Ca²⁺ exchanger indicating that it may be a member of the superfamily of this exchanger. The coupling of this exchanger to the trans neuronal Na⁺ gradient and the putative stoichiometry of this transporter, 3 Na⁺ per one Zn²⁺, indicate that even a partial collapse in the Na⁺ gradient such as observed during ischemia or seizure may trigger a reversal in its cation transport and similarly to the Na⁺/Ca²⁺ exchanger this will lead to enhanced influx of Zn²⁺ further exacerbating intracellular Zn²⁺ load under these conditions.

1.3. ZIP family

Members of the ZIP (solute carrier 39) family of transporters are key players in mediating Zn²⁺ influx from the plasma membrane and across intracellular compartments. Much less is known about their catalytic mechanism compared to ZnTs. Previous studies have indicated that ZIP are acting as Zn²⁺/HCO₃⁻ co-transporters [42, 43]. More recent studies on the bacterial ZIP homologue that was purified and reconstituted into proteoliposomes indicate that they are acting as heavy metal channels that are permeable to both Zn²⁺ and Cd²⁺ [44]. They further showed that these proteins are pH regulated, strongly activated by acidic pH. Such pH regulation is consistent with

their role in regulating Zn²⁺ level at acidic intracellular organelles and indicates that the H⁺ in these compartments may have a physiological role in regulating Zn²⁺ transport via ZIP.

ZIP transporters are ubiquitously expressed in brain [45]. Their potential role is particularly interesting in this organ but only very recent studies have started to address their role in the brain. For example, zinc uptake in neuroblastoma cells is regulated by the expression of ZIP6 and is affected by polarization status of these cells [46]. The ZIP4 membrane expression in neurons is enhanced following excitotoxic insults [47]. Remarkably, interaction of the serine protease plasminogen activator tPA appears to enhance zinc influx mediated by ZIP4 leading to enhanced lysosomal sequestration of Zn²⁺. Moreover, enhanced prosurvival signaling linked to the interaction of tPA and ZIP4 indicates that it has a prosurvival effect on neurons. There are still many unsolved questions regarding the physiological role of ZIP proteins in the brain. For example, which members of the ZIP family are participating in Zn²⁺ uptake from the synaptic cleft.

2. Major Stores of Zinc

Zinc is found in two major pools in the brain. One is a complex form with other proteins [48] and the other is the vesicular "free" Zn²⁺ that is unique to the brain and some secretory cells [49], like the pancreatic β-cells [50]. The first pool of Zn²⁺ was considered inert for many years, but an accumulating body of evidence shows that this Zn²⁺ is responsible for both signaling and Zn²⁺ toxicity [25, 51, 52].

2.1. The Dynamic Nature of the Bound Zinc Pool

The bound Zn²⁺ could be found tightly bound to zinc finger proteins, or in complex with cysteine residues in numerous proteins, most prominent are the metallothioneins (MT). In agreement with the inherently low redox potential of MT proteins, several works have shown that even mild thiol oxidation or nitrozilation is sufficient to induce liberation of Zn²⁺ from these proteins. Indeed, various oxidative stimuli and NO-related species were shown to induce this toxic surge of intracellular Zn²⁺ [25-27, 53-57]. MTs are widely expressed in the brain and have an important role in protection and regeneration of neurons and glia [58], MT-1 and MT-2 are found glia cells and neurons, albeit with lower expression, while MT-3 is exclusively found within neurons [59, 60]. Interestingly, MT-3 is also associated with regions where the synaptic Zn²⁺ pool is found. While neurons contain many zinc transport proteins, as discussed above, a role for MT-3 is probably to buffer the cytoplasmic Zn²⁺ concentration following its influx into the cytoplasm. Interestingly, the levels of this protein are highly regulated by intracellular Zn²⁺ levels [61]. Finally, cellular organelles as mitochondria may contain "releaseable Zn²⁺", which induces a rise in the cytoplasmic level of this ion following excitotoxic activity [62].

2.2. Signaling by Synaptic Zinc

The synaptic Zn²⁺ pool was first identified in the hippocampus, where it is still most studied, already in the 1950's when zinc-dithiazone exhibited bright staining [63, 64].

Future works repeatedly showed staining of the mossy fibers vesicles with the free form of this ion [65, 66], and the zinc transporter 3, ZnT3, was identified as the protein responsible for this accumulation of zinc [67, 68]. Importantly, developmental regulation of ZnT3 protein was found in conjunction with the accumulation of synaptic Zn^{2+} . Moreover, ZnT3 KO animals were shown to lack synaptic Zn^{2+} pools, yet intracellular Zn^{2+} rise and toxicity was observed in these mice, suggesting that the toxic Zn^{2+} rise may originate from the bound-zinc pool within the cells [69]. As mentioned above, it is now known that the ZnT3 KO mice are more susceptible to kainite induced seizures [22] and adult animals show impairment in learning and memory function [31]. Synaptic Zn^{2+} is mostly found in glutamatergic vesicles [70-72], not only the mossy fibers but also in the amygdaloidal pathways that project into the cerebral cortex and subcortical targets [73, 74]. It has been estimated that about half of the glutamatergic synapses within the cerebral cortex and limbic regions contain chelatable Zn^{2+} . Several different independent approaches were used to show the release of this synaptic Zn^{2+} pool. The first studies were based a push-pull canula and indicated that Zn^{2+} is released at high concentrations, in the mM range, following activation of the mossy fibers [75]. Studies employing $^{65}Zn^{2+}$ analysis in brain slices yielded similar values for Zn^{2+} released in the CA3 region [76]. Further measurements employed fluorescent zinc sensitive dyes to monitor the release of this ion into the extracellular region in the CA3. Interestingly, this Zn^{2+} release was attenuated in younger animals in accordance with the developmentally regulated accumulation of this ion [77]. Further works have shown that such Zn^{2+} release occurs following physiologically relevant stimulation of the mossy fibers and can be used as a reliable marker for the release of vesicular glutamate [78, 79]. It is important to note that these measurements require careful choice of the Zn^{2+} sensitive probes, for example, the Kd of the probe should be in the low mM range so that the concentrations in the synaptic cleft region will not saturate it [80]. Indeed, while the overwhelming majority of the studies suggested the release of synaptic zinc, it was also claimed that the Zn^{2+} produces a veneer effect, in which it is externalized but does not fully dissociate from the vesicle but sticks to the neuronal membrane [81]. These findings were however challenged by subsequent studies demonstrating that synaptic zinc release is also observed by low affinity Zn^{2+} sensitive dyes and clearly monitored even following a single synaptic release event [78, 82, 83]. Thus based on overwhelming large number of studies employing a wide range of approaches it is now well accepted that the synaptic Zn^{2+} is indeed released and reaches postsynaptic cleft regulating the post synaptic receptors. How much Zn^{2+} is released is still a matter of debate. Postsynaptic receptors have well defined and specific sites for Zn^{2+} that by interacting with these receptor modulates their activity [84-88]. Because the affinities of these sites to Zn^{2+} is well established they can be used as effective endogenous reporters for synaptic Zn^{2+} levels. Thus for example, functional analysis of the Zn^{2+} effect on the NMDA receptor indicate that Zn^{2+} may reach hundreds of micromolar levels [89]. Future studies based on synaptic zinc in situ probes will be however required to firmly establish these values (see chapter 9).

3. Zinc in Epilepsy

The vesicular zinc pool is found in limbic regions prone to seizure [49] and thus a role for this zinc in epilepsy has been suggested. Based on the effects of Zn^{2+} on ionotropic receptors, the vesicular zinc pool has been suggested as proconvulsive or

anticonvulsive. The majority of the studies, however, showed an anticonvulsive role for the vesicular zinc pool. The ZnT3 KO mice, lacking synaptic Zn^{2+} , are a useful genetic model to study the role of this pool, as they exhibit rather normal synaptic transmission [21]. In fact, the first abnormal phenotype trait observed in these mice is the enhanced susceptibility to kainite-induced seizure [22]. In another genetic model of ethanol withdrawal, synaptic Zn^{2+} deficiency was also linked to enhanced seizure susceptibility [90]. Chemical chelation of the synaptic Zn^{2+} , using the high affinity chelator DEDTC or a low affinity chelator Clioquinol, resulted in a similar effect of overexcitability and enhanced seizure susceptibility [91, 92]. Dietary zinc-deficiency that leads to loss of synaptic Zn^{2+} levels, also enhanced susceptibility to kainite-induced seizures [93, 94]. Moreover, addition of Zn^{2+} via direct infusion into the brain, delayed development of discharges in a kindling model [95]. It should be noted that one study in contrast showed that chelation of zinc reduced seizure activity triggered by intensive electrical stimulation of the amygdala [96]. Finally, anticonvulsive effects of synaptic zinc may also be documented in some forms of human epilepsy where zinc chelation has been associated with acute seizure and possibly even temporal lobe epilepsy [97, 98]. In addition, neonatal seizures have been linked to zinc deficiency [99].

Following the epileptic seizures and neuronal loss reorganization of the neuronal circuitry occurs [100]. In the hippocampus, this process results in enhanced plasticity of the non-injured neurons and extensive mossy-fiber sprouting observed in experimental models or humans. These fibers, rich in vesicular zinc, form synapses on the inner molecular layer of the dentate gyrus enhancing the excitatory drive and were thought to contribute to repeated seizures and epileptogenesis [101, 102]. Other studies suggested in contrast, that the recurrent fibers to innervate the inhibitory neurons in this region and thereby induce a hyperinhibitory effect [103, 104]. Changes in Timm's staining following seizure are used to monitor the neuronal sprouting in the hippocampus and also in the amygdalar regions [105]. While a functional study suggested a lack of effect for the recurrent fibers-synaptic Zn^{2+} another study suggested that the recurrent fibers Zn^{2+} pool induces facilitation of epileptiform activity, and application of the extracellular Zn^{2+} chelator, CaEDTA reduced evoked bursting activity [106]. During seizures, excessive release of zinc together with glutamate has been monitored in the hippocampal mossy fiber region [93]. In accordance, 24h following kainite injection loss of the vesicular zinc pool is observed in all brain regions rich in vesicular zinc, hippocampus, amygdale and cerebral cortex [93]. A longer follow up of zinc levels following induction of status epilepticus revealed dramatic reduction of the vesicular zinc for up to one month in the amygdala, after which a recovery phase that peaks at 3 months after the initial insult is observed [107].

The mechanism by which zinc may affect seizure activity is still not clear. Early studies suggested a role for Zn^{2+} in allosterically modulating the ionotropic receptors for GABA, NMDA and glycine in vitro [108]. Indeed, Zn^{2+} interacts with specific binding sites on these receptors and different affinities of such binding sites have been described. Inhibition of the NMDA receptors by Zn^{2+} , for example, is mediated by a high affinity binding site on the NR2A subunits suggesting that *in vivo* Zn^{2+} may exert both tonic and phasic inhibition of this receptor [109, 110]. Glycine receptor dependent currents are enhanced by low Zn^{2+} concentrations and inhibited by higher Zn^{2+} concentrations, but the former mechanism seems to be relevant following synaptic Zn^{2+} release in the spinal cord [87]. Finally, Zn^{2+} inhibits the GABA_A receptors [111, 112] but also enhances release of GABA via a pre-synaptic mechanism [113]. Thus, via attenuation of the GABAergic inhibitory drive one can imagine a proconvulsive role

for synaptic Zn^{2+} , while an anti-convulsive effect of Zn^{2+} may be mediated by the increased GABA release. However, a clear effect of synaptic Zn^{2+} , as opposed to exogenous Zn^{2+} application, was not yet clearly observed on any of the ionotropic pathways. A promising pathway for synaptic Zn^{2+} in modulation of neuronal excitability and seizure activity is linked to the recently identified Zn^{2+} -sensing receptor, ZnR [114]. This receptor is found on postsynaptic neurons in the hippocampal CA3 region and is activated by synaptic Zn^{2+} released following mossy fiber stimulation. Activation of several kinase pathways triggered by the ZnR signaling, among them most notably MAPK and CaM kinase, is linked to the regulation neuronal excitability [115, 116]. Thus activation of the ZnR may constitute an important part of the mechanism of synaptic Zn^{2+} in epilepsy, which is consistent with the general fundamental role of metabotropic receptors in epilepsy [117]. Another intriguing pathway is the trk receptor, central to neuronal survival but also to long term potentiation and emerging as a critical player in epilepsy [118]. Studies of neuronal cultures found that extracellular Zn^{2+} , by activating metalloproteinases, facilitate the activation of the trk ligand BDNF leading to trk receptors activation [119]. Subsequent studies on brain slices suggest that Zn^{2+} regulated trk by entering the postsynaptic neurons in the CA3 region activating trk leading to enhanced long term potentiation of these neurons [120]. Hence these newly discovered pathways provide very promising handles for studying the well-known but poorly understood effects of synaptic Zn^{2+} in neuronal excitability and the etiology of seizure.

4. Zinc Homeostasis and Signaling in Glia

The presence of chelatable Zn^{2+} pools in neurons but not in glia, as well as the general interest in neurons shifted the focus of the Zn^{2+} field to neurons. In addition, early studies have indicated that the concentration of Zn^{2+} required to kill glia cells are much higher and perhaps not at the physiological range of Zn^{2+} found in the brain [121]. This view was dramatically changed in recent years and Zn^{2+} has been linked to numerous physiological and pathophysiological aspects function of glial cells as well as their interaction with neurons.

4.1. Regulation of Ca^{2+} Channels

Calcium is the major second messenger of glial signaling mediated by agonists such as ATP and endothelin [122]. The store operated channel (SOC) is the major pathway for Ca^{2+} influx in glia that is activated by the depletion of the intracellular Ca^{2+} stores. Interestingly, SOC activity is blocked in the presence of Zn^{2+} indicating that Zn^{2+} is a potent regulator of this Ca^{2+} influx pathway [123]. Notable, Zn^{2+} inhibits the SOC with a K_1 of 6 μ m, a concentration well within the physiological concentration of free Zn^{2+} in the brain. Consistent with such role, depletion of the intracellular Ca^{2+} following repetitive application of agonists was accelerated by the presence of Zn^{2+} , indicating that by regulating this pathway, Zn^{2+} can control the Ca^{2+} content and rate of depletion of the stores [124].

The L-type Ca^{2+} channel (LTCC) is also prominent Ca^{2+} influx pathway in astrocytes. In contrast to SOC that is permeable to Ca^{2+} but not to Zn^{2+} , the LTCC is highly permeable to Zn^{2+} and is a major pathway for toxic Zn^{2+} permeation into astrocytes [3]. Remarkably, pretreatment with sublethal Zn^{2+} concentration, which

enhances expression of ZnT1, protects astrocytes from subsequent toxic Zn²⁺ insults. The protective effect of Zn²⁺ preconditioning was followed by a marked decrease in Zn²⁺ influx through the LTCC indicating that ZnT1 may act as a regulator of this cation permeation pathway. Thus, a preconditioning effect that can result from relatively small changes in extracellular Zn²⁺, induced by mild episodes of seizure, may protect the brain from subsequent massive increase in Zn²⁺. Further modulations of ion channels by Zn²⁺ are described in chapter 7.

4.2. Release of Zn²⁺ from Intracellular Stores

Similar to the effects of cellular Zn²⁺ rise in neurons, subsequent studies suggested that a rise in intracellular Zn²⁺, particularly the release of the Zn²⁺ that is bound to MTs, may also play a critical role in glial death. This release of Zn²⁺ is particularly sensitive to oxidative or nitrosative stress in astrocytes similar to this process in neurons. In astrocytes this Zn²⁺ rise, induced by the release of this ion from metallothionein 3, may regulate the lysosomal function and induce autophagy [125-127]. Indeed, the induction of autophagy in astrocytes was followed by massive fusion of autophagic vacuoles (AV) with lysosomes. Chelation of Zn²⁺ using TPEN was followed by a reduction in the number of AV vesicles while exposure to Zn²⁺ increased their number, indicating that Zn²⁺ dyshomeostasis is linked to autophagy (see chapter 5). Finally, chelation of Zn²⁺ or inhibition of autophagy blocked the lysosomal membrane permeabilization (LMP). Because lysosomes contain numerous potentially harmful enzymes the enhanced LMP is a critical step in cell death, and the strong inhibitory effect of Zn²⁺ chelation on LMP highlights the critical role of Zn²⁺ in oxidative induced astrocytic, as well as neuronal [128] cell death. The rise in astocytic Zn²⁺ levels was also shown to induce PARP-1 activation leading to impairment of glutamate uptake by the astrocytes [129]. This finding suggests that during oxidative stress when intracellular Zn²⁺ level is increased astrocytes may fail to support glutamate uptake and may contribute further to neuronal injury.

Astrocyte swelling is an early and important phase of a wide range of brain disorders, leading to brain edema induced by stroke, or liver dysfunction. The latter injury leads to brain accumulation of ammonia that triggers, in turn, astrocytes swelling and brain edema. Astrocytes swelling induced by hypo-osmotic solution or ammonia triggers a robust rise in intracellular Zn²⁺ that can be blocked by NMDA antagonists, antioxidant or NO synthase blockers [130]. In contrast to neurons, such Zn²⁺ rise in astroglia did not trigger their death but instead induced the expression of the heavy metal master regulator MTF1 and MT [3] indicating that Zn²⁺ may enhance the expression of protecting protein that counteract massive swelling or oxidative stress. Similarly, the release of Zn²⁺ in astrocytes is triggered by acidosis often linked to brain ischemia and may also trigger the expression of Zn²⁺-dependent genes [131].

4.3. Zn²⁺ Uptake by Endocytosis in Glia

Fluorescent and electron microscopy analyses indicate that Zn²⁺ uptake in astrocytes is mediated by an endocytotic pathway as inhibitors of the endocytotic machinery leads to the inhibition of Zn²⁺ uptake [132]. Electron microscopy analysis further demonstrated that Zn²⁺ uptake in astrocytes is followed by its accumulation in lysosomes. In accordance, it was suggested that ethanol reduces astrocytic Zn²⁺ levels via its effects on membrane trafficking [133]. Further work is required to identify whether specific

transporters are involved in astrocytic Zn²⁺ uptake and how do they interact with the endocytic pathway to transport Zn²⁺ into lysosomes.

4.4. Glia-Neuron Interaction

Glial neuronal interaction is emerging as one of the important aspects of brain physiology. However the role of Zn²⁺ in this interaction is still poorly understood.

Extracellular Zn²⁺ has a pleotropic effect of enhancing activation of microglia. At physiological concentrations of 15-30 μM Zn²⁺, this ion enhances NF-KB activity and triggers cytokine expression and is essential to induce the activated morphology of microglia via the NADPH [134]. Moreover, Zn²⁺ injected into mouse brain induced microglia activation while chelating Zn²⁺ with CaEDTA suppressed microglia activation induced by cerebral ischemic reperfusion insult. Thus, during excitotoxic conditions when neuronal cells are dying, the release of the Zn²⁺ from these cells may activate the neighboring microglia cells. Interestingly, intracellular Zn²⁺ rise can not only induce neuronal and glial death but is also a critical step by which microglia kill neurons [135]. Microglia, activated by neuronal injury, further induce neuronal death and are considered a major factor in neuronal degeneration in numerous brain disorders. Massive release of nitrogen and oxygen radical is a hallmark of activated microglia. An elegant study has demonstrated that these species induce the liberation of Zn²⁺ in neighboring neurons leading to enhanced K⁺ currents that trigger neuronal apoptosis [136]. The lethal link between microglia and neurons could be eliminated by scavenging of radicals or by Zn²⁺ chelation. Notably overexpression of MT3 confers a similar neuronal protection against microglial induced cell death. A recent work has shown that a rise in microglia Zn²⁺ levels, induced by metamphetamine, may modulate the activation of the P2X Ca²⁺ channels and thereby exacerbate neuronal demise [137]. Further studies are required to identify the receptor by which extracellular Zn²⁺ activates the microglia and the pathways which allow Zn²⁺ permeation into these cells. Nevertheless, an intriguing vicious cycle in which microglia, by killing neurons, will be further activated by the released Zn²⁺ is already evident from the literature.

Interestingly, crosstalk between neurons and glia cells has also been shown to be mediated by metallothioneins (MT). It has been shown that following exposure of astrocytes to Zn²⁺ in the presence of IL-1 MTs are secreted by these cells and are interacting with neurons [138]. The released MTs can interact with specific receptors megalin on neurons that then induces internalization of the MTs. This process is followed by accelerated axonal regeneration following toxic nerve injury. Further studies are required to elucidate the signaling pathways in which the uptake of MT enhances neuronal regeneration and if the neuronal uptake of MT is also followed by a change in Zn²⁺ content in the neuronal cells.

The results obtained in recent years highlight the importance of glial cells in brain zinc homeostasis. They clearly indicate that in contrast to the belief that glial cells are inert to Zn²⁺ they mediate highly dynamic Zn²⁺ homeostasis. They further suggest that glial Zn²⁺ homeostasis is critical for their function and is involved in glial neuronal crosstalk. The availability of sensitive Zn²⁺ probes and our understanding of neuronal glial interaction will certainly facilitate the discovery of many additional aspects by which these cells interact and the pathways via which these interactions occur.

5. Conclusion and Perspectives

Studies in the last 20 years revolutionized our view on brain zinc homeostasis and its physiological role. Using specific Zn²⁺ sensitive dyes these studies have identified two major Zn²⁺ pools, synaptic and cellular, with distinct physiological roles. Other studies identified signaling pathways linking a change in Zn²⁺ in distinct cellular compartments to neuronal activity and brain damage. Identification of specific Zn²⁺ binding sites on the major neuronal receptors led to mechanistic insight on the role of Zn²⁺ in synaptic transmission and neuronal function. Interestingly a specific target for the synaptic Zn²⁺, the ZnR, was identified and may affect neuronal excitability under physiological conditions. Glial cells once thought to play a minor role are emerging as active players in Zn²⁺ homeostasis. A knock down model of the ZnT3 transporter provided an invaluable tool for investigating the role of synaptic Zn²⁺ in a wide spectrum of neuronal processes and brain disorders ranging from learning processes to seizure ischemia and AD. Identification of the major Zn²⁺ transporters and receptors as well as their mode of action and distribution, in health and disease, will provide a molecular basis linking Zn²⁺ homeostasis and signaling to physiological and pathophysiological processes in the brain.

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19. Zinc and Cortical Plasticity

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Abstract. In addition to the milieu of systems this nutritionally essential element contributes to in the human body, the divalent cation zinc also participates in neuronal signalling within the nervous system. Specifically, a subset of zinc within the brain is located in the synaptic vesicles of some glutamatergic neurons and can be released at the presynaptic terminal in an activity-dependent manner. The location of zinc releasing neurons and pathways, and the co-release of synaptic zinc with neurotransmitters such as glutamate, supports the involvement of synaptic zinc in neuromodulation and, specifically, in cortical plasticity. In fact, chelation of extracellular zinc *in vitro* has been demonstrated to inhibit LTP, while sensory deprivation or sensory stimulation in intact animals can lead to activity-dependent increases or decreases of synaptic zinc, respectively.

Keywords. Cerebral cortex, synapse, learning, memory, neurotransmitter, neuromodulator

Introduction

The neuronal circuits of the mammalian cerebral cortex are in a state of perpetual flux, retaining mutable properties even in adulthood. These alterations in the morphology and physiology of the cerebral cortex are broadly labeled as “plasticity” and are often the result of changes in the sensory environment of an organism. Cortical plasticity is generated and maintained by several different factors that act in concert. While extensive research has illuminated many of the major components and processes involved in cortical plasticity, much remains to be elucidated. Numerous lines of evidence derived from anatomical, electrophysiological, and behavioural studies indicate that the divalent cation zinc is involved in the processes of cortical plasticity.

1. Zinc Homeostasis within the Brain

Before discussing the involvement of zinc in cortical plasticity, it would be beneficial to discuss how zinc homeostasis is maintained. Alterations in zinc levels outside of normal physiological concentrations have been implicated in epilepsy, ischemia, and neurological diseases such as Alzheimer’s and depression [1; 2]. As such, cellular zinc homeostasis is strictly maintained within the mammalian cells in general and in neurons specifically. The cellular influx, efflux, and transport of zinc are maintained by three classes of proteins. These are, Zrt-Irt-like proteins (ZIP), metallothioneins (MT), and zinc transporter (ZnT).

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1.1. Zinc Influx

ZIP transporters are responsible for the influx of zinc into the cytoplasm. The ZIP family transporter 1 (hZIP1) is ubiquitously expressed in all cells including neurons. hZIP1 is located on the plasma membrane as well as the endoplasmic reticulum and facilitates the transport of zinc into the cell [3]. Thirteen other mammalian ZIP transporters have been identified. All except ZIP7, which is located at the Golgi apparatus, were observed at the plasma membrane. However, the characterization, precise locations of expression, and exact mechanism for zinc translocation for all fourteen ZIP transporters have yet to be outlined in detail (see chapters 8 and 28).

1.2. Intracellular Zinc Levels

The cytoplasmic concentration of zinc within the neuron is buffered by metallothioneins. MTs are able to bind up to seven zinc atoms and thus act as a buffer, binding zinc atoms temporarily at high zinc concentrations and releasing zinc atoms when it is low (see chapter 4). Four MTs have been characterized in mammals. Of these, MT-I and MT-II can be found in several cell types including astrocytes and spinal glia, while MT-IV is found in some types of epithelial cells [4]. MT-III on the other hand is exclusive to neurons, and particularly to neurons containing synaptic zinc. Perhaps as a consequence to its association with synaptic zinc, MT-III has been linked to the pathophysiology of neurological diseases. It has been suggested that MT-III is down regulated in Alzheimer's and MT-III knock out mice have increased susceptibility to kainic acid induced seizures [5; 6].

Mitochondria may act as another modulator of intracellular stores of zinc since zinc influx and efflux has both been observed in the mitochondrion [7]. Although submicromolar concentrations of zinc are enough to cause mitochondrial dysfunction [8], under normal circumstances, intracellular free zinc ion concentrations are low or even estimated by some to be non-existent [9]. In concert with MTs, mitochondria likely facilitates as a buffer for cytoplasmic zinc.

1.3. Efflux of Cytosolic Zinc

The transport of zinc out of the cytoplasm within mammalian cells depends on a family of zinc transporters (ZnT). ZnTs are responsible for the efflux of cytosolic zinc to clear excess zinc and transport zinc into intracellular organelles. Within the neuron, ZnT1 facilitates zinc efflux at the plasma membrane while ZnT-2 and 4 package zinc into endosomes and lysosomes [10]. A total of 10 ZnT proteins have been described. Their precise function, however, remain to be characterized, especially in the neuron.

ZnT-3 is the most thoroughly characterized ZnT protein and is of particular interest when considering neurons. ZnT-3 is responsible for the movement of cytosolic zinc into synaptic vesicles [11]. The deletion of this protein in ZnT-3 knockout (KO) mice leads to a complete lack of synaptic zinc within the brain.

2. Synaptic Zinc

Approximately 20% of zinc in the brain is found within synaptic vesicles [12]. This encapsulated, synaptic pool of zinc allows for its visualization through the formation of zinc-selenite precipitate with sodium selenite and further staining with silver lactate [13; 14]. As previously mentioned, ZnT3 is responsible for the movement of cytoplasmic zinc into synaptic vesicles. The distribution of ZnT3 within the brain follows that of synaptic zinc and knockout of ZnT3 corresponds to the loss of approximately 20% of zinc within the brain and complete loss of synaptic and histochemically reactive zinc [12].

2.1. Localization of Synaptic Zinc

Synaptic zinc is co-localized with γ -aminobutyric acid (GABA)-ergic, glycinergic, and glutamatergic neurons [for review see 15], while zincergic neurons within the brain are almost exclusively also glutamatergic [14; 16]. Although not all glutamatergic neurons contain synaptic zinc, zincergic neurons comprise an estimated 50% of all glutamatergic synapses in some regions [17].

These zincergic neurons are found in high concentrations throughout the cerebral cortex and within limbic structures such as the hippocampus, amygdala, and olfactory bulb [18]. Within cortical and limbic structure layers, the distribution of synaptic zinc is heterogeneous, concentrated in lamina that are rich with dendrites and synapses – layers I-III, V, VI – while very low in thalamic recipient layers – layer IV [19]. Zincergic neurons project exclusively within corticocortical, corticolimbic, and limbic-cortical circuits [18-20]. This suggests that synaptic zinc is implicated in higher order functioning as well as synaptic plasticity within these synapse-rich layers and evolutionarily recent and, arguably, more complex networks.

2.2. Synaptic Zinc Release

The localization of zinc in synaptic vesicular pools and its co-localization with glutamatergic terminals both invite the assumption that synaptic zinc may be released in an activity-dependent manner into the synaptic cleft. Earlier studies employing high, non-physiological stimulation of mossy fibre in hippocampal slices provided support for zinc exocytosis [21; 22]. The development of zinc fluorescence imaging and electrophysiological recordings further provided strong evidence for quantal zinc release following single action potentials within both hippocampal mossy fibre and CA1-CA3 synapses [23].

An increase in extracellular zinc concentration induced by zinc exocytosis, however, has been reported to vary from nM to as high as 300 μ M levels [for review see 24]. This disparity in measurements of zinc concentration may be caused by differences between the age of the animal used, the use of zinc chelators, and the temperature of the tissue slice preparation across different labs [25]. Despite the lack of consensus regarding the concentration of zinc that is released and the observation of zinc externalization in intact animals, the majority of evidence supports the activity dependent synaptic release of zinc.

3. Zinc and Synaptic Plasticity

The co-release of synaptic zinc with glutamate and the near exclusively intra-cortical distribution of glutameric pathways infer a relationship between zinc and cortical plasticity. Synaptically released zinc can modulate purinergic, nicotinic, glycinergic, GABA, serotonin, and dopamine receptors [for review see 15]. Zinc's effects on these receptors are often specific to the receptor subunits and will vary depending on the concentration of extracellular zinc measured. The general direction of zinc's effect is inhibitory at higher but physiologically relevant concentrations (~100 μ M to 300 μ M) and possible potentiation at lower concentrations (~10 μ M). Chelation of endogenous extracellular zinc, on the other hand, can be pro-convulsive, as seen with intracranial administration of zinc salts [26].

3.1. NMDA Receptor Inhibition

Upon initial inspection, the effect of zinc on the glutamate sensitive N-methyl-D-aspartic acid (NMDA) receptor follows this general rule. Zinc is largely a non-competitive allosteric, voltage-independent inhibitor of channel opening probability [27; 28]. The wealth of research performed on the relationship of zinc with NMDA receptors further revealed zinc concentration-dependent modulation of NMDA receptor function that is specific to NMDA receptor subunit composition [for review see 24]. Zinc does not ubiquitously affect all NMDA receptors and the elicited response is attributed to NMDA receptor 2 (NR2) subunits. The binding site for zinc on NR2 precedes the glutamate-binding domain and is located on the N-terminal domain of both NR2A and NR2B receptor subtypes [29; 30]. No binding site for zinc exists on NR2C and NR2D.

At nM concentrations, zinc is a high affinity voltage-independent inhibitor of NR2A. 1~10 nM of zinc added to the extracellular bath can attenuate up to 80% of postsynaptic NMDA receptor activity in HEK and *Xenopus* oocytes [28]. At μ M concentrations, zinc inhibition of NR2A channel opening probability becomes voltage-dependent. 10~100 μ M of zinc at negative membrane potentials can increasingly reduce NR2A receptor activity as the membrane potential drops [31; 32]. An additional, intra-channel NR2A binding site for zinc at asparagine N595 is likely responsible for this voltage-dependent inhibition. Inhibition of NR2B by zinc, on the other hand, occurs only at 1~10 μ M zinc concentrations. This blockage of NR2B-containing NMDA receptor current is voltage-independent as well as nearly complete [28; 32]. These nM and μ M concentration-dependent effects suggests that zinc may exert tonic as well as phasic modulation of NMDA receptor activity within the brain.

Prolonged exposure to zinc at higher concentrations (0.3 mM ~1 mM for 10 min) can, alternatively, potentiate excitatory postsynaptic potentials (EPSPs) mediated by both NR2A and NR2B-containing NMDA receptors within the hippocampus [33]. It was also observed that this potentiation is mediated by the activation of Src family tyrosine kinase and an increase in tyrosine phosphorylation of both NR2A and NR2B subunits. The co-localization of synaptic zinc with glutamatergic neurons within the brain and the faceted modulation of NMDA receptor activity by zinc both infer a very possible implication of synaptic zinc in synaptic plasticity. Zinc modulation of Src family tyrosine kinase implicates possible tropomyosin-related kinase (Trk) B activation and further strengthens a role for zinc in cortical plasticity.

Indeed, physiological levels of zinc ($10 \mu\text{M}$ for < 15 min) can initiate the release of pro-brain derived neurotrophic factor (pro-BDNF) from mouse astrocyte cultures as well as the conversion of pro-BDNF to mature BDNF through the extracellular activation of metalloproteinases, thereby transactivating Trk pathways [34]. Zinc-initiated activation of the Trk receptor pathways can also occur in an intracellular and BDNF-independent manner. Zinc can transactivate TrkB signalling through increasing Src family tyrosine kinase activity [35]. This activation of Src family tyrosine kinases by zinc is itself secondary to the high affinity binding and inhibition of C-terminal Src kinase by zinc [36]. C-terminal Src kinase promotes the autoinhibition of Src family tyrosine kinase and, when inhibited by zinc, lead to an increase in Src family tyrosine kinase activity and subsequent transactivation of TrkB. The mutual activation shared between Src family tyrosine kinase and TrkB [37] further places zinc as the multi-pathway upstream promoter of TrkB signaling.

Zinc's subunit-specific modulation of NMDA receptor function may yet prove to be more fruitful in describing the involvement of synaptic zinc in cortical plasticity. There are for instance, similarities between the maturation of NMDA receptor expression and the synaptic zinc response to sensory manipulation. Mature neurons primarily express NR2A over NR2B subunits [for review see 38]. The progressive increase in NR2A subunit expression in the cortex begins after birth, critically inverts to more NR2A than NR2B subunits at postnatal day (P) 7, and stabilizes at roughly P15 [39]. This timeline coincides with the pattern of synaptic zinc staining following sensory deprivation. An increase in zinc staining following long-term deprivation (3 weeks) can only be detected in mice younger than P8, while short-term deprivation (< 48 hours) only leads to zinc staining increase when administered to mice older than P15 [40; 41].

The ability for rapid metaplasticity, changes in the relative expression of NR2A and NR2B subunit, of NMDA receptors in response to inhibition at the single synapse level [42] may also point to a possible downstream effect of synaptic zinc. The possibility of a zinc receptor within hippocampal neurons mediated by G-protein-coupled receptor 39 (GPR39) further provides a novel pathway for synaptic zinc modulation of neuronal function [43].

4. Synaptic Zinc and Cortical Plasticity

As mentioned, zinc can have a number of effects on a wide variety of neuronal signaling mechanisms, both intra- and extracellularly. The capacity of zinc to act as a multifaceted neuromodulator is indicative of its involvement in the expression and maintenance of plasticity. Experiments that have detailed how synaptic zinc is involved in cortical plasticity have examined many different components of plasticity, ranging from the examination of behavioural deficits to signaling at single synapses. While an extensive examination of these studies is not possible, a brief summary of the literature concerning these domains is warranted.

4.1. Zinc and Behaviour

4.1.1. Dietary Manipulations of Zinc

Zinc deficient and rich diets have been utilized as a method to systemically manipulate zinc concentrations. Dietary zinc deficiency in early life, achieved by feeding mothers

zinc-deficient diets both pre- or postnatally, can result in learning and memory deficits in the offspring [44-47]. Zinc deficient diets in adulthood can lead to learning deficits as well and these animals can exhibit behaviours similar to that of stressed animals [48; 49]. Interestingly, zinc supplementation can also affect behaviour; performance on the Morris water maze is impaired following such a manipulation [50]. It is important to note, however, that since zinc is involved in so many biological processes, dietary manipulations of zinc will affect a large number of systems. Indeed, it is unclear if changes in dietary zinc levels lead to any changes in synaptic zinc levels [51].

4.1.2. Genetic manipulations of synaptic zinc

In order to more clearly examine how alterations in synaptic zinc levels can affect behaviours, mice with genetic mutations or manipulations can be employed. The *mocha* mouse is the result of a spontaneous mutation [52]. This mutation was found to inactivate AP-3, which, among other processes, can affect the positioning of ZnT3 onto synaptic vesicles [53; 54]. As such, *mocha* mutant mice are found to have significantly lower synaptic zinc levels in the brain. These mice have behavioural abnormalities such as aberrant motor and coordination abilities, hyperactivity, and enhanced auditory gating, indicating that changes in synaptic zinc levels can affect behaviour [52; 55].

The *mocha* mutant is an imprecise model for examining behaviour in an organism with reduced synaptic zinc because the inactivation of AP-3 can affect other factors such as the packaging of chloride channels [56]. A powerful model with which to study the effect of synaptic zinc on behaviour is the ZnT3 KO mouse, which, as mentioned, lacks the transporter responsible for loading zinc into synaptic vesicles and therefore, has no synaptic zinc. Surprisingly, initial study revealed no major behavioural deficits in this mouse. Behavioural measures of sensorimotor ability, anxiety, and leaning and memory all indicated that the KOs performed comparably to controls [57]. The only phenotype that was observed was an increase in the susceptibility of the KO mice to kainic acid induced seizures and a resistance to seizures induced by bicuculline [58].

While initial examination into the behavioural characteristics of the ZnT3 KO mouse were disappointing and perplexing, subsequent studies have revealed a behavioural phenotype in these mice. Moreover, these deficits are observed in tasks requiring cortical plasticity. By using aged mice (6 months of age), deficits in learning and memory, as revealed by the Morris water maze, were observed in ZnT3 KO mice [59]. Interestingly, these changes were accompanied by alterations in the levels of neuronal proteins. Specifically, significantly decreased expression of SNAP-25, PSD-95, TrkB, Pro-BDNF, DCX, AMPA receptors, and the NR2A and NR2B subunits of the NMDA receptor was observed, while the expression of synaptophysin increased [59]. Additionally, deficits were observed in younger (3-5 months) ZnT KO mice, including deficits in fear conditioning and extinction, spatial memory, and contextual discrimination [60; 61]. The deficit in contextual memory observed in ZnT3 KO mice can be mimicked through the localized chelation of synaptic zinc in wildtype animals, indicating that this effect is the result of the absence of synaptic zinc [60]. Thus, mice lacking synaptic zinc do have behavioural deficits related to cortical plasticity and additionally, have many biochemical changes that may mediate these changes.

4.2. Zinc and LTP/LTD

Electrophysiological investigations into the role of zinc in cortical plasticity have focused primarily on the hippocampus, given the high levels of synaptic zinc found there. Electrophysiological substrates of plasticity, long-term potentiation and depression (LTP and LTD, respectively), are markedly affected by alterations in zinc concentrations in the mossy fibre and Schaffer collateral pathways [35; 62-65]. The common finding is that levels of zinc in excess or depletion of zinc through chelation or dietary restriction both inhibit the induction of LTP. These findings are not surprising given that the concentration of zinc is a crucial factor when considering how it can affect neurotransmission. Demonstrative of this notion, Izumi and colleagues [66] illustrated how different concentrations of zinc could affect LTP and LTD in a manner that was dependent on the subunits composition of NMDA receptors. Their results suggested that the binding of zinc to the NR2B subunit of NMDA receptors by zinc resulted in an inhibition of LTD induction while LTP inhibition resulted following the binding of zinc to both binding sites for the ion on the NR2A subunit.

In addition to the modulation of NMDA receptors, zinc has been shown to affect plasticity through other processes as well. As mentioned, it has been demonstrated that zinc can affect the activity of the TrkB signaling pathway [35]. Activation of TrkB is required for LTP to occur at the mossy fibre synapse and this activation is dependent on the presence of zinc. It was theorized that released synaptic zinc entering the postsynaptic neuron through NMDA receptors could affect the intracellular signaling cascade integral for the activation of TrkB. The activation of TrkB could then initiate signaling mechanisms that would result in a retrograde messenger being released, leading to an increase in glutamate release and the expression of LTP [35].

In the lateral amygdala, the removal of zinc through the use of a chelator has been shown to abolish LTP. LTP could be restored through the use of a GABA_A antagonist, indicating that the effect was the result of zinc was acting on GABA_A receptors. Since synaptic zinc is not found in cortical GABAergic neurons, it was postulated that zinc spillover from glutamatergic neurons was responsible [67].

The electrophysiological properties of the ZnT3 KO mouse have been examined and, much like the initial behavioural characterization, results were disappointing. No differences between KO and WT mice were observed in a number of measures including evoked field potentials, spontaneous excitatory postsynaptic potentials, GABA_A or GABA_B receptor mediated responses, or NMDA and non-NMDA receptor mediated responses [68]. However, no published study has examined LTP and LTD in the ZnT3 KO. Additionally, given the expression of a behavioural phenotype in older ZnT3 KO mice, it is important that these electrophysiological measures be examined in aged mice as well.

4.3. Experience-Dependent Regulation of Synaptic Zinc

It is apparent that the concentration of synaptic zinc present is a critical factor regarding how zinc can affect cortical plasticity. While behavioural and electrophysiological studies have primarily examined how adding or removing zinc to preparation can affect measures of plasticity, the inverse can also be investigated. Namely – how the occurrence of plasticity can affect levels of synaptic zinc. To do this, researchers have utilized sensory manipulations that give rise to large and predictable plastic changes in the respective sensory cortex and assessed changes in synaptic zinc levels afterward. In

the visual cortex of non-human primates, short-term (24 hours) monocular deprivation results in an increase in synaptic zinc levels within the ocular dominance columns corresponding to the deprived eye. Long-term (3 months) deprivation, interestingly, results in a decrease in synaptic zinc levels within the deprived ocular dominance columns [69]. While the non-human primate visual system is ideal for studying such an effect, as cortical plasticity changes can be easily mapped to the ocular dominance columns, the use of such subjects is often not feasible for most researchers. Instead, the majority of studies examining the experience-dependent regulation of synaptic zinc have utilized the rodent vibrissae sensory system.

Rodents obtain much of their sensory information about the environment through their vibrissae or whiskers. As such, a large portion of the somatosensory cortex is devoted to processing information from the whiskers and is organized both functionally and morphologically in a manner as to mimic the distribution of the whiskers. The “barrel cortex”, as this region is known, is a useful system in that there is a one-to-one correspondence between a whisker and a cortical barrel such that sensory manipulations on a single whisker can be accurately mapped to a specific region of cortex. Removal of whiskers has been shown to lead to a robust increase in synaptic zinc levels within the corresponding barrels, even in adult animals [40; 70-72]. This increase is rapid, being observed as quickly as 3 hours following the manipulation, and correlates to the degree of whisker re-growth [70]. Stimulation of whiskers, conversely, results in a decrease in synaptic zinc levels within the corresponding barrels [73].

The bi-directional alteration of synaptic zinc levels in response to manipulations of sensory input is considered to be evidence of the involvement of synaptic zinc in cortical plasticity. However, it is sometimes argued that these changes in synaptic zinc levels are an epiphenomenon; simply, a passive increase or decrease in synaptic zinc levels due to changes in neuronal activity that are unrelated to the process of plasticity. This is likely not the case for two reasons. First, the experience-dependent regulation of synaptic zinc has been shown to be dependent on age. The magnitude of alterations in synaptic zinc levels in older mice is significantly less than that observed in young mice [74; 75]. Mice housed in enriched environments, on the other hand, are found to have elevated zincergic responses to whisker removal [76]. If the experience-dependent regulation of synaptic zinc were an epiphenomenon, these results would suggest that aged mice are better able to maintain zinc homeostasis and that mice housed in enriched environments are less able. This is likely not the case. Rather, it is more probable that the decrements in cortical plasticity that occur with aging as well as the alterations that occur with enrichment are responsible for this effect.

Second, ultrastructural examination of the experience-dependent regulation of synaptic zinc has indicated that this effect is at least partially mediated by synapses changing their “phenotype”. The increase in synaptic zinc following whisker plucking could be the result of three, non-mutually exclusive mechanisms. There could be an increase in the number of synapses that contain synaptic zinc, an increase in the number of vesicles containing zinc per synapse, and/or an increase in the amount of zinc contained within vesicles [73]. To assess the first possibility, the number of synaptic zinc containing synapses was counted in barrels undeprived and deprived of sensory input. A significant increase in the number of synapses containing synaptic zinc was observed and this change occurred independent of a corresponding increase in the number of excitatory synapses [77]. This result indicates that pre-existing synapses can come to newly house synaptic zinc, which is contradictory to the notion that there is merely a passive process leading to the accumulation of synaptic zinc.

Interestingly, this study also observed changes in synaptic zinc within layers II/III and V wherein which alterations are not observed in response to sensory manipulations at the light microscopy level [77]. This is likely because there is such a high concentration of synaptic zinc, such that it is difficult to resolve any changes. These layers are more plastic in adulthood compared to layer IV [78], where most zincergic changes are observed, and thus, this observation lends further evidence to support a role for synaptic zinc in cortical plasticity.

4.4. Functional Implications for Plasticity Resulting from Alterations in Synaptic Zinc Levels

It is apparent that cortical plasticity is not a unitary phenomenon and can be mediated by many factors and can take many forms. In addition to the original “Hebbian” model, wherein certain activity patterns are conducive to an increase or decrease in synaptic efficacy, other conceptualizations of plasticity exist. Homeostatic plasticity is one such example, where changes in synaptic function are observed as a mechanism to maintain synaptic efficacy or “gain” [79; 80]. Whatever the model that underlies the expression of plasticity, it is important to recognize that plasticity, broadly defined, are changes in synaptic function. Such changes do not necessarily have to result in alterations in synaptic efficacy. As such, any changes observed in the large assortment of factors involved in neurotransmission can be considered plasticity. The experience-dependent regulation of synaptic zinc can therefore be regarded as a form of plasticity in itself. What remains unknown is how this phenomenon interacts with other factors involved in neurotransmission, resulting in the expression of cortical plasticity.

The possible mechanisms through which alterations in synaptic zinc levels could affect cortical sensory maps are numerous. Following sensory deprivation, if one assumes that the increase in synaptic zinc levels corresponds to an increase in zinc release, it is conceivable that this change in the amount of zinc released would have implications for neurotransmission and plasticity. Within the broad schemes of Hebbian and homeostatic plasticity, it is easy to speculate how the experience-dependent regulation of synaptic zinc would fit. The increase in released zinc could affect postsynaptic receptors as to facilitate LTP or LTD-like processes, generating cortical activity changes that are Hebbian in form. Conversely, an increase in the level of synaptic zinc following sensory deprivation may be a form of homeostatic plasticity. An increase in the amount of zinc that is released by a particular stimulus may be a process of synaptic scaling to maintain activity at a certain level. This process could potentially be pre- or postsynaptically mediated.

Released synaptic zinc is likely able to modulate many forms of plasticity, probably through a multitude of processes. An obvious candidate is the NMDA receptor, which, as mentioned, is potently modulated by zinc [28; 31; 81; 82]. Changes in the activity of the NMDA receptor could potently affect the expression of cortical plasticity. Additionally, an increase in the level of zinc in the synaptic cleft may facilitate the entry of zinc into the postsynaptic neuron through the NMDA channel, calcium permeable AMPA channels, or voltage gated calcium channels [83-86]. Once inside the postsynaptic neuron, this zinc could affect a number of signaling molecules such as protein kinase C, calcium calmodulin kinase II, or cyclic AMP, which are implicated in the expression of cortical plasticity [87-89]. As mentioned, activation of the TrkB signaling pathway is important for some types of plasticity. Although some discrepant results have been obtained as to the mechanisms through which zinc can

modulate TrkB signaling, the fact that zinc does affect this signaling pathway indicates that synaptic zinc could modulate cortical plasticity through this process [33-35; 37; 90].

Alternatively, the involvement of synaptic zinc in cortical plasticity may be derived from its other effects on other neurotransmitter systems. As discussed above, the spillover of released zinc has been speculated to affect cortical GABAergic neurons in a manner as to influence plasticity [67]. Such spillover could allow released zinc to modulate a number of different neurotransmitter signaling. For example, zinc can inhibit the K⁺/Cl⁻ co-transporter 2 (KCC2), a transporter responsible for ensuring that GABAergic activity is inhibitory [91]. While this effect may be more pertinent to situations of hyperactivity, even slight adjustments in activity through such a route could be important for plasticity. Moreover, acetylcholine signaling is an important modulator of cortical plasticity and zinc can affect the function of nicotinic receptors [92; 93].

A relatively new avenue through which synaptic zinc could affect cortical plasticity is GPR39 or the “zinc receptor” [43; 94]. The binding of zinc to this receptor leads to the activation of mitogen activated protein kinase (MAPK) and CaMKII pathways, both of which are involved in cortical plasticity [43; 95; 96]. Although much remains to be elucidated concerning the function of the GPR39 receptor in both neurotransmission and cortical plasticity, the existence of such a receptor is telling as to the capability of synaptic zinc to alter neurotransmission.

5. Conclusion and Perspectives

At present, it is clear that the lack of synaptic zinc can affect learning and memory behaviour, that changes in zinc levels can affect a variety of neurotransmitter systems and therefore, LTP and LTD, and that cortical plasticity is accompanied by alterations in synaptic zinc levels. However, these studies must be linked together in order to generate a conclusive conceptualization for the role of synaptic zinc in cortical plasticity. In the absence of these empirical investigations, one can only speculate on the precise function of synaptic zinc and the specific mechanisms by which it can affect cortical plasticity. Nevertheless, in the context of what is currently known about the neurobiology of zinc, it is evident that synaptic zinc is intimately involved in the process of cortical plasticity.

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20. Zinc and Mental Health

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Abstract. Depression is a chronic recurring illness that is associated with significant disability, morbidity and mortality. Depression is twice as prevalent in women as men and the risk factor for prenatal depression and postpartum depression is similar to the risk factor for depression in general. Attention deficit hyperactivity disorder (ADHD) is a developmental disease, characterized by overactivity, impulsivity and inattentiveness. ADHD affects about 5-9% of children aged between 5 and 14 years and 2-3 times as many boys as girls. The presence of ADHD in childhood is a major risk of the development of neurobehavioral disorder in adults. Despite the intensive research of both diseases, the exact mechanisms involved in the pathophysiology and treatment of depression or ADHD is still unknown.

This paper is focused mostly on depression and ADHD in the context of the role of zinc deficiency in the pathogenesis of these diseases, changes in animal behavior, the role of zinc treatment and zinc supplementation, and possible biological mechanisms involved in these relationships.

Keywords: Zinc, Depression, Anxiety, ADHD, Schizophrenia

Introduction

There are many preclinical and clinical data reported the key role of zinc in the pathophysiology of such mental diseases as depression, ADHD or schizophrenia.

For depression, animal studies focus mostly on two fields of research: the antidepressant-like properties of zinc in different tests and models of depression, and behavioral changes after zinc deficiency. In turn, clinical research includes alterations in the serum zinc concentration in depressed patients and the effects of zinc supplementation in the treatment of depression.

For ADHD, the animal studies focus on the effect of zinc deficiency on the activity or attention processes but clinical studies can be divided into three major fields of research: 1) the level of zinc in the blood, hair or urine of children with ADHD; 2) zinc as a supplementary medication or adjunct to the psychostimulant therapy in the treatment of ADHD and 3) the relation between the efficacy of psychostimulants and the zinc level.

It is also suggested that alterations in zinc level or zinc availability may be related to the pathophysiology of schizophrenia.

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The present review summarizes the most important data concerning the role of zinc and zinc deficiency in the development or treatment of depression, ADHD and schizophrenia.

1. Zinc and Depression

1.1. Zinc Level in Depression (Table 1)

Clinical studies indicated that subjects suffering from major depression and unipolar depression showed significantly lower serum zinc levels than non-depressed controls [1-3]. However, no alterations in blood zinc level in depressed patients were also reported [4;5]. Interestingly, patients with minor depression (with either dysthymic disorder or an adjustment disorder featuring a depressed mood) showed intermediate zinc levels [1;6] when compared to healthy volunteers. A lower serum zinc level was also found to accompany antepartum and postpartum depressive symptoms [7]. Data published by Siwek et al 2010 [8] showed that low zinc concentration accompanies acute depression symptoms and that a low zinc level was negatively correlated with the duration of the depression episode. It was also found that treatment-resistant patients demonstrated a lower concentration of zinc than treatment-non-resistant patients [8]. Although the results concerning the zinc level in depression are rather consistent in all of the studies, certain discrepancies appear among data on the correlation between zinc and the severity of depression. In two groups of patients, with either major depression or postpartum depression, a significant negative correlation between zinc concentration and the severity of depression was observed [1;3;7]. However, in two other papers, by Maes 1997 and Siwek et al. 2010 [8,9], this correlation was not found. The authors suggest that this discrepancy seems to be related to the differences in patients enrolled for the study; especially treatment-resistant patients [8]. Low zinc level was also found in depressed patients with end-stage renal disease on hemodialysis [10]. The other important results obtained from clinical trials include the effects of antidepressants on the serum zinc level. It was found that successful antidepressant therapy can lead to a normalization of the zinc level [2;6;9;10;11]. The other data published by Siwek et al. 2010 [8] showed that in non-resistant patients the zinc level increased when therapeutic response or remission was achieved, while in treatment-resistant, placebo treated patients the zinc level either did not change from the initial value or only slightly increased during the treatment. Very recently, Sussulini et al. 2010 [12] reported an increased zinc level in bipolar patients treated with lithium compared to healthy controls.

Our previous [13] and unpublished present studies conducted in suicide victims and compared with psychiatrically normal controls showed no differences in the zinc concentration either in the hippocampus or in the frontal cortex. However, we observed a statistically significant decrease in the ability of zinc to inhibit the [³H]MK-801 binding to N-methyl-D-aspartate (NMDA) receptor ionophore in the hippocampus (but not in the frontal cortex) of suicide victims when compared to control subjects [13]. This data may indicate that the alterations in the interaction between zinc and NMDA may be involved in the psychopathology underlying suicidality. The other group examined zinc concentration in brains of suicidal victims suffered of bipolar depression. They demonstrated reduced zinc concentration in frontal cortex (but not in the other brain region) in bipolar depressed suicidal victims compared with control subjects [14].

1.2. Zinc Deficiency (*Table 1*)

Most of the data concerning dietary zinc intake and depression come from animal studies and suggest a causative role for zinc deficiency in the induction of depressive and anxiety-like behavioral symptoms. Studies on zinc-deficient mice showed increased immobility time in the forced swim test (FST) and tail suspension test (TST) and increased anxiety-related behavior in the novelty suppressed feeding test, measured as enhanced latencies to eat [15;16]. Enhanced depression-like behavior, induced by zinc deficiency, found in the FST and the TST was reversed by desipramine (DMI) treatment [16]. Other studies found that rats fed on a zinc-deficient diet displayed anhedonia, anorexia and an increase in anxiety-like behavior observed in a light-dark box test [17;18]. Zinc deficiency was also found to enhance the aggressive behavior of young mice elicited by social isolation [19].

There are only a few clinical studies that have examined the relationship between dietary zinc intake and depression symptoms. One study, published by Amani et al., 2010 [¹], was taken from 23 young women diagnosed with moderate to severe depression and 23 age-matched healthy volunteers. All of the women involved in this study completed a semi quantitative food frequency questionnaire containing the main food sources of zinc in the dietary patterns and a 24-h-food recall questionnaire to assure the daily zinc intakes. It was found that both the daily zinc intake and the serum zinc level in the major depressive group were about two thirds of that observed in the healthy group. Moreover, an inverse correlation was found between the serum zinc concentration and depression scale scores [²]. The other study investigating the relationship between dietary zinc intake and depression in the context of the role of psychosocial stress and sociodemographic factors in the development of depressive symptoms was conducted using a group of pregnant women from London [20]. In this study, the depressive symptoms were evaluated using the Center of Epidemiologic Studies Depression Scale (CES-D) and the zinc intake was quantified based on the food frequency questionnaire and nutrient supplement data. Analysis of the results showed that lower zinc intake, higher stress and social disadvantage were associated with higher CES-D scores and that higher zinc intake attenuates stress-induced depressive symptoms [20].

The relationship between zinc status, nutritional aspects and the psychological dimensions in the old subjects required in Northern and Southern European Commission was the topic of the ZINCAGE project [21]. 853 old healthy subjects, classified into four different groups of age were screened for nutritional status (Frequency Food Questionnaire), cognitive status (Mini Mental State Examination, MMSE), perceived stress (Perceived Stress Status, PSS) and mood (Geriatric Depression Scale, GDS). The results showed that 82% of total samples showed no cognitive decline, 72% exhibited a rather low GDS value which indicates no depression and normal perceived stress level (whole samples). But what is interesting, this study showed a relevant relationship between all psychological dimensions studied in project and plasma zinc values or nutritional assessment but not age, which suggest that an adequate zinc intake may be useful to maintain a satisfactory psychological status in elderly and healthy ageing [21].

1.3. Antidepressant Effect of Zinc (Table 1)

Many studies using rats and mice have examined the antidepressant-like properties of zinc in animal drug screening tests and models of depression. Zinc administration induced an antidepressant-like effect (reduction in immobility time) in both the FST and TST [22-27]. Zinc was also active in different models of depression: olfactory bulbectomy (OB) (reduction in numbers of trials in the passive-avoidance test and a decreased OB-induced hyperactivity in rats) [26]; chronic mild stress (CMS) (reversion of the CMS-induced reduction in the consumption of sucrose) [28] and chronic unpredictable stress (CUS) (preventing deficits in the fighting behavior of chronically stressed rats) [29]. It is worth emphasizing that zinc not only exhibits antidepressant-like effects in the rodent tests and models of depression when it is given alone but also intensifies the effects of standard antidepressants in these behavioral experiments. The synergistic effects of zinc and other antidepressants such as IMI, fluoxetine, paroxetine, bupropion or citalopram were seen in the FST, the TST and CUS [22;27;29-31]. Moreover, our unpublished data indicates the anxiolytic-like activity of zinc in rodent tests.

There are also some clinical studies that showed that zinc supplementation may enhance antidepressant therapy. The pilot study of zinc supplementation in antidepressant therapy, described in the paper of Nowak et al., 2003 [32], was conducted in patients who fulfilled the DSM IV criteria for major (unipolar) depression. Patients received zinc supplementation (25mg Zn/day) or placebo and were treated with standard antidepressant therapy (tricyclic antidepressants, selective serotonin reuptake inhibitors). The patient's status was evaluated before the treatment and 2, 6 and 12 weeks after the treatment using the Hamilton Depression Rating Scale (HDRS) and Beck Depression Inventory (BDI). In this study, antidepressants reduced HDRS scores by the second week of treatment in both groups and BDI scores at the sixth week in the zinc-treated group. Zinc supplementation significantly reduced scores in both measures after 6- and 12-weeks of supplementation when compared with the placebo treatment. A beneficial effect of zinc as an adjunct agent was also found in treatment-resistant patients [33]. This placebo-controlled, double blind study of zinc supplementation in imipramine therapy was performed in 60 patients aged between 18 and 55, fulfilling the DSM-IV criteria for major depression without psychotic symptoms. The patients were randomized into two groups: the first were treated with imipramine (140mg/day) and received one daily placebo while the second were treated with imipramine and supplemented with zinc (25mg Zn/day) for 12 weeks. It was found that zinc supplementation significantly reduced the depression scores (measured by Clinical Global Impression [CGI]; Montgomery-Asberg Depression Rating Scale [MADRS]; BDI and HDRS) and facilitated the effect of the treatment in antidepressant treatment resistant patients. No significant differences in CGI, MADRS, BDI and HDRS scores were demonstrated between zinc and placebo-supplemented antidepressant treatment non-resistant patients. DiGirolamo et al., 2010 [34] examined the effect of zinc treatment on different children's behavioral measures. They demonstrated that, while there were no differences between the zinc and placebo treated groups, zinc treatment increases the serum zinc level, which was inversely associated with a reduction in depressive and anxiety symptoms. The paper, published recently by Sawada and Yoki, 2010 [35] indicated that zinc supplementation may have potential to prevent of depressive symptoms. In this study, young women taking multivitamins and zinc showed a significant reduction in depression and anxiety

symptoms when compared to women taking only multivitamins. However, the other study by Nguyen et al., 2009 [36] reported no significant reduction in depression scores with zinc supplementation.

1.4. Effect of Antidepressant Treatment on Brain Zinc

The first experimental data of the effect of antidepressant treatment (drugs and electroconvulsive shocks, ECS) were simultaneously published in 1999. A histochemical study using Timm's method (a method imaging the presynaptic-vesicle pool of zinc) demonstrated that chronic ECS treatment increases the vesicular zinc level in rat hippocampus [37]. The other article demonstrated that chronic ECS highly increased total zinc concentration in rat hippocampus, while the effect of imipramine and citalopram was low in this measure [38]. The next follow up papers confirmed the ECS-induced enhancement of hippocampal vesicular zinc level without effect of fluoxetine or desipramine [39;40]. In the recent report we found (using a modified Timm's method) that chronic treatment with citalopram and imipramine increases the pool of vesicular zinc in the rat prefrontal cortex but not in the hippocampus [41]. This report also examined extracellular zinc pool (determined by the anodic stripping voltammetric method in brain microdialyzates) and showed an increase of the extracellular zinc level in the prefrontal cortex but not in the hippocampus after chronic citalopram and imipramine treatment. Moreover, chronic treatment with zinc induced enhancement in zinc level in both brain structures and in both measures. All the data indicates that brain zinc is involved in the mechanism of antidepressant treatment.

1.5. Biological Mechanisms

The best established mechanism involved in the antidepressant-like activity of zinc is the inhibitory modulation of glutamate signaling through the inhibition of NMDA receptors or enhancement of α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) glutamate receptors [42;43]. It was found, for example, that the antidepressant-like effect of zinc observed in the FST was abolished by N-methyl-D-aspartate acid, which acts as a specific agonist at the NMDA receptor, or by the D-serine cotreatment, which is the agonist of the glycineB site of glycine/NMDA receptors [44;45;46]. The antidepressant effect of zinc observed in the FST was also abolished by NBQX (an AMPA receptor antagonist) and the synergistic effect of both the AMPA receptor potentiator CX614 and zinc was found in the FST [46]. The other possible mechanism of zinc's action in depression is the modulation of the serotonergic system. In fact, zinc was found to increase the density of 5-HT1A and 5-HT2A receptors in the rat hippocampus and frontal cortex, respectively [45] and the synergistic effect of zinc with "serotonergic" antidepressants was shown in the rodents' tests [22;24;27;30;31]. The third suggested mechanism of zinc action is the regulation of the brain-derived neurotrophic factor (BDNF) level. It was found that chronic zinc treatment increases the BDNF gene expression and protein level in the rat's brain [23;28;47].

Table 1. Clinical data of the involvement of zinc in depression.

Reference	Clinical data
Placebo controlled trials of zinc treatment/supplementation in depression	
Nowak et al., 2003 [32]	Zinc supplementation (daily dose: 25mg Zn ²⁺ as zinc hydroaspartate + tricyclic antidepressants or selective serotonin reuptake inhibitors; length 12 weeks) - reduced HDRS and BDI scores
Siwek et al., 2009 [33]	Zinc supplementation (daily dose: 25mg Zn ²⁺ as zinc hydroaspartate + ~140mg/day imipramine; length 12 weeks) - reduced depression scores and facilitated treatment effect in antidepressant treatment resistant patients
DiGirolamo et al., 2010 [34]	Zinc treatment (daily dose: 10mg zinc oxide, 5d/week, length 6 months) - no effect on depressive and anxiety symptoms in children
Sawada and Yokoi., 2010 [35]	Zinc supplementation (daily dose: 7mg/kg; zinc gluconate + multivitamins, length 10 weeks) - reduced depression and anxiety symptoms in young women
Nguyen et al., 2009 [36]	Zinc supplementation (daily dose: 20.2mg/kg; zinc sulfate +folic acid, iron and vitamin B-12, length 12 weeks) - no relationships between depression and zinc
Blood zinc concentration in depression	
Low level	
Maes et al., 1994 [1]	48 unipolar depressed patients (16 minor, 14 simple major, 18 melancholic subjects) and 32 healthy volunteers.
McLoughlin and Hodge, 1990 [2]	14 patients with primary affective disorder and 14 age-and sex-matched controls.
Nowak et al., 1999 [3]	19 unipolar depressed patients and 16 healthy volunteers.
Maes et al., 1997 [9]	31 unipolar depressed patients and 15 healthy volunteers.
Wojcik et al., 2006 [7]	66 pregnant women (66 were assessed for prepartum depressive symptoms and 62/58 women for postpartum depressive symptoms)
Amani et al., 2010 [6]	23 major depressed female patients and 23 healthy female volunteers
Siwek et al., 2010 [8]	60 major depressed patients (40 females, 20 males) and 25 healthy volunteers (16 females and 9 males)
Roozbeh et al., 2010 [10]	103 depressed and 32 non-depressed patients with end-stage renal disease on hemodialysis
No alteration	
Narrang et al., 1991 [5]	35 depressed patients and 35 healthy volunteers
Irmisch et al., 2010 [4]	88 depressed patients (56 females, 32 males) and 88 healthy volunteers (56 females and 32 males), possible effect of antidepressant treatment
DiGirolamo et al., 2010 [34]	674 children, serum zinc level was inversely associated with depressive and anxiety symptoms
Effect of antidepressant treatment on blood zinc level	
McLoughlin and Hodge, 1990 [2]	Increased (normalization) of low serum zinc
Schlegel-Zawadzka et al., 2000 [11]	Increased (normalization) of low serum zinc
Sussulimi et al., 2010 [12]	Increased of serum zinc
Maes et al., 1997 [9]; Siwek et al., 2010 [8]	Increased (normalization) of low serum zinc after successful therapy Not increased low serum zinc in treatment resistant patients

2. Zinc and ADHD

2.1. Zinc Deficiency (*Table 2*)

Both the animal and clinical studies suggest the important role of zinc deficiency in ADHD. Halas and Sandstead, 1995 [48] published the data suggesting the involvement of zinc deficiency in hyperactivity in rats. The other study discovered reduced motor activity and impairment of attention in moderately zinc-deprived monkeys at the level that did not cause growth retardation or a reduced plasma zinc level [49-51].

Several clinical studies have shown that children with hyperactivity have lower zinc concentrations than healthy children. These studies were carried out in children from different countries and the zinc level was measured in different samples such as: urine, hair, plasma, and erythrocytes. One of the studies conducted in Poland [52] demonstrated a low average concentration of zinc in 50 children (aged from 4-13 years) with hyperactivity when compared to control-healthy children, with the highest zinc deficit being observed in hair. In the other Polish study, a high rate of zinc deficiency was found in 116 children with ADHD in comparison to the control group [53], and what is interesting is that ADHD children with comorbid disorders exhibit a lower hair zinc level than children with ADHD alone [53]. In the next study, performed in Turkey, a low serum zinc level was reported in 48 children diagnosed with ADHD when compared to 45 healthy volunteers [54]. Additionally, in the ADHD group; a significant correlation was found between zinc and low free fatty acid (FFA) levels, which are also considered to be important factors in influencing hyperactivity [54]. A lower serum zinc level was also found in ADHD children from Israel when compared to the control children [55]. In 13 ADHD children the zinc level was between 4,76 -8.3 $\mu\text{mol/L}$ while the normal serum zinc level is about 13-15 $\mu\text{mol/L}$ [55]. The next study, comprising 44 British children with ADHD aged between 6-12 years, showed that 66% of these children are deficient in zinc [56]. A low serum zinc level (about 30% of the laboratory reference range) was found in 48 American children in the ages of 5-10 years. In this study, the serum zinc level correlated with parent-teacher-rated inattention [57]. Another study published by Yorbik et al., 2008 indicated that low plasma zinc level observed in children with ADHD might have an effect on information processing in children with hyperactivity [58].

2.2. Zinc Supplementation (*Table 2*)

The important role of zinc in ADHD is supported by several studies revealing that zinc can be a beneficial treatment for children with this disease. A double-blind, placebo-controlled study covering 400 children with ADHD (328 boys and 72 girls) showed significant improvement in hyperactivity, impulsivity and socialization symptoms after zinc sulfate treatment, as assessed by Attention Deficit Subscales (ADHDS), although zinc treatment had no effect on attention deficits [59]. Interestingly, older children with higher body mass indexes (BMI) and low levels of zinc and FFA responded best to the zinc treatment [59]. The other double-blind, placebo-controlled study, performed in a group of 44 Iranian patients with ADHD (26 boys and 18 girls), that viewed the effect of zinc plus methylphenidate, demonstrated the beneficial effect of zinc as a supplementary medication in the treatment of ADHD [60]. Children treated with combined therapy, such as zinc and methylphenidate, showed a significantly greater improvement in parent and teacher rated ADHD symptoms than the placebo group,

who received only methylphenidate by itself [60]. However, these studies seem to be somewhat limited when compared to what is suggested by other authors [34;61]. Firstly, these studies were carried out in an area where there was a suspected endemic zinc deficiency; and, secondly, different measures are used to obtain the data and high dropout after randomization and a too high dose of zinc being points raised for Bilici et al., 2004 study [59]. Some studies have suggested the relationship between the response to psychostimulants and zinc concentration. A placebo-controlled study conducted in boys with ADHD showed that the effect of amphetamine treatment was related to nutritional zinc concentration and the best effects were observed in the children with an adequate zinc level [62;63]. However, there is also some data that did not indicate any beneficial effect of zinc supplementation in the improvement of psychosocial or behavioral functioning. A study, using school-age children in Guatemala as its source, showed no effects of six months' zinc supplementation on their mental outcomes (hyperactivity) [34]. No effects of zinc supplementation in the improvements in the ratings of behavior were also observed in lead-exposed Mexican children [64]. Recently, Arnold et al., 2011 [65] published results of the study examined the effect of zinc supplementation in American children with ADHD. In this study zinc does not improve inattention activity more than placebo, however it was found that optimal amphetamine dose with zinc supplementation was lower than with placebo.

These results indicate that more research in well diagnosed patients is needed to clearly define the value of zinc supplementation in ADHD.

2.3. Possible Biological Mechanisms of Zinc in ADHD

Many studies suggest that symptoms of ADHD are linked to imbalances in the dopamine neurotransmission in the prefrontal cortex. Stimulants such as methylphenidate and amphetamine, which are commonly used as a first-line drug in the treatment of ADHD, increase the availability of dopamine [61;66]. In fact, its effects are related to the inhibition of the dopamine reuptake transporter, which limits not only the intensity of dopamine but also the duration of the action of this neurotransmitter at pre and postsynaptic receptors [61;67;68]. Several studies show that the human dopamine transporter contains an endogenous high-affinity zinc-binding site on its extracellular domain and that the binding of zinc on the dopamine transporter leads to non-competitive inhibition of the dopamine translocation (outward/reverse transport) [69;70]. As such, this data suggests a "psychostimulants-like" profile of zinc action and can explain the beneficial effect of zinc supplementation in methylphenidate treatment of ADHD. The second possible mechanism involved in the psychostimulants-like effect of zinc is its commitment in the production and modulation of melatonin, which helps regulate the function of dopamine [71;72]. In fact, it is proposed that there is some relation between dosing the time-dependent action of psychostimulants and the melatonergic system in the treatment of ADHD [61;72;73;74].

Table 2. Clinical data of the involvement of zinc in ADHD.

Reference	Clinical data
Placebo controlled trials of zinc treatment/supplementation in ADHD	
Bilici et al., 2004 [59]	Zinc treatment (daily dose: 150mg zinc sulfate; length 12 weeks) - reducing symptoms of hyperactivity, impulsivity and impaired socialization (ADHD scale)
Akhondzadeh et al., 2004 [60]	Zinc supplementation (daily dose: 55mg zinc sulfate +1mg methylphenidate; 6 weeks) - improvement in parent and teacher rated ADHD symptoms
DiGirolamo et al., 2010 [34]	Zinc supplementation (daily dose: 10mg chewable zinc; 6 months) - no effect on mental health outcomes;
Kordas et al., 2005 [64]	Zinc supplementation in lead-exposed Mexican children (30mg/day zinc oxide; length 6 months) - no effect
Arnold et al., 2011 [65]	Zinc supplementation in American children (15mg/day or 30mg/day as zinc glycinate; length 8 weeks) – no effect
Low blood zinc concentration in ADHD	
Kozielec et al., 1994 [52]	50 Polish children with hyperactivity; age 4-13 years
Starobrat-Hermelin, 1998 [53]	116 Polish children with ADHD;
Bekaroglu et al., 1996 [54]	48 Turkish children diagnosed with ADHD; 45 healthy volunteers
Toren et al., 1996 [55]	ADHD children from Israel
Kiddie et al., 2010 [56]	44 British children with ADHD; age 6-12
Arnold, 2005 [57]	48 American children (37 boys, 11 girls; 33 combined type, 15 inattentive); age- 5-10 years
Yorbik et al., 2008 [58]	28 boys diagnosed with ADHD and 24 healthy boys

3. Zinc and Schizophrenia

It is suggested that alterations in zinc level might also be related to the pathophysiology of schizophrenia. Rahman et al., 2009 [75] have demonstrated decreased hair concentration of zinc in the group of 30 male schizophrenic patients when compared to 30 healthy control subjects. However, in the study published by Yanik et al., 2004 [76], the plasma zinc concentration did not differ between schizophrenic patients (39) and healthy controls (34). The findings of previous research on the status of zinc level in patients diagnosed with schizophrenia were controversial too and both lowered and unchanged zinc concentration was observed. It is suggested that these discrepancies between findings might result from a different patient populations or different laboratory methods used in the studies [see [76].

The other hypothesis about the role of zinc in schizophrenia was associated with the learning and memory deficits observed in schizophrenia and related to regional alterations in the volume of the hippocampus. Since many critical pathways in the hippocampus containing ionic zinc, so it was suggested that the alterations in

hippocampal innervations observed in schizophrenia may be associated with a decrease in ionic zinc. In fact Goldsmith and Joyce 1995 [77] found significantly lower zinc level within the hippocampal mossy fiber system in the brain of schizophrenics than in healthy controls [77]. However, the other study performed in postmortem hippocampal tissue from schizophrenic patients and matched control subjects found comparable levels of ionic zinc in both groups [78]. These data are consistent with the results published by Kornhuber et al., 1994 [79] showing no difference in total hippocampal zinc level in postmortem brains between controls and schizophrenic patients.

Alternate hypothesis suggested the role of zinc in pathophysiology of schizophrenia is called a Gestational Zinc Deficiency Theory. That theory indicates that schizophrenia is caused by the action of gestational zinc deficiency on genetically susceptible fetuses [80] and psychosis commonly observed in schizophrenia are the effects of the combination of dietary induced zinc deficiency and lack of zinc releasing capacity in the hippocampus [80].

Genetic studies indicate that schizophrenia might be partly caused by mutations in a gene encoding a protein involved in the zinc transport. One protein suggested as a candidate gene is gene encoding a solute carrier family 39 (zinc transporter) member 12. Study performed in DNA obtained from postmortem brain tissue from schizophrenics and unaffected individuals showed that a missense homozygous mutation in exon 2 (A213G resulting in S36G) occurred about twice often in the schizophrenics group as in control [81].

4. Conclusion and Perspectives

The data mentioned in this paper does not give a clear answer to whether zinc deficiency is the cause of depression, ADHD or schizophrenia or is the result of these diseases. However, some evidence should be illustrated: many patients with depression, and children with ADHD, have a lower zinc level, indicating either that zinc levels could be a marker of these diseases or, where depression is concerned, it could be a marker of treatment resistance. It is also suggested that adequate zinc nutritional status may improve the response to commonly used drugs or may lower the dose of drugs needed for its benefit. Since zinc deficiency perpetuates several negative neurobiological and behavioral consequences, the problem of general malnutrition should be taken into consideration.

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21. Zinc and Alzheimer's Disease

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Abstract. The maintenance of normal zinc levels is crucial to a variety of cellular functions, with zinc also shown to be a key mediator of a number of central and peripheral disorders. Within the brain, there is now mounting evidence for a pivotal role for zinc dyshomeostasis in the onset and pathological and symptomatic progression of Alzheimer's disease- the most common form of dementia. In this chapter we will discuss the evidence for a disturbance in zinc levels in both the brain and peripheral compartments in Alzheimer's disease. We will further outline the various cellular targets that may be affected by this and how it may contribute to the pathophysiology of Alzheimer's disease. Finally, we will discuss the notion that zinc dyshomeostasis may represent a therapeutic target for this progressive neurodegenerative disorder.

Keywords. Zinc; Alzheimer's disease; Amyloid; Therapeutic.

Introduction

Zinc is an essential element within the healthy brain, facilitating many normal cellular processes/functions as outlined in earlier chapters. Following a perturbation in zinc levels, or the occurrence of disease or external stimuli (such as head trauma), many of these zinc-dependent processes may become dysregulated to then initiate or propagate a pathological state within the brain. In this chapter we are going to focus on the role of zinc in Alzheimer's disease (AD).

Alzheimer's disease is a chronic neurodegenerative disorder that, following the first description in 1906 and subsequent publication in 1907 by Alois Alzheimer [1], now represents the most prevalent form of dementia in modern society, accounting for between 60-80% of all dementia cases [2].

The clinico-pathological features of AD have been extensively described elsewhere [1, 2]. Briefly, individuals with AD have a symptomatic presentation that involves progressive cognitive decline that ultimately also erodes all higher order executive functioning. On post-mortem examination these individuals exhibit a number of cardinal features within the brain, the most pronounced of which is the presence of extracellular deposits referred to as "plaques" and intracellular accumulations referred to as "neurofibrillary tangles" (NFTs). Other gross manifestations include the thinning of the cortical grey matter, the enlargement of the ventricular spaces and a generalised atrophy of the brain. Microscopic examination also reveals a characteristic loss of synaptic connections, gliosis and the presence of tortuous neurites throughout the neuropil and particularly in association with the plaques. While there are many more

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clinical and pathological features found in AD, it is the presence of cerebral plaques and NFTs that is required for the post-mortem diagnosis of AD.

The potential role for zinc in dementia was first proposed by Burnet [3], and since that time there has been an ever increasing amount of research into the role of metals in AD which has expanded our understanding of both the pathogenesis of AD and the potential therapeutic avenues for this disease. In this chapter we review the evidence supporting a role for metals, in particular zinc, in a number of the key features of AD.

1. Altered Zinc Levels in Alzheimer's Disease

The change in zinc levels with age has been covered in earlier chapters. However, to briefly reiterate, the concentrations of zinc within the brain tend to rise from birth to adulthood and then remain stable throughout life within a reasonably narrow concentration range [4]. Similarly, zinc levels in the plasma tend to remain constant for most of adult life, up until advanced age when they decline [5-11].

In AD there have been a variety of investigations into the zinc content of multiple different tissues, including the CSF, blood and brain. Within these studies, particularly those of the brain, it is important to distinguish between studies that undertook bulk tissue studies and those that undertook an analysis of fractionated/region-specific tissues. A previous ICPMS study has demonstrated that even the normal brain is characterised by significant region-specific variability in metal content [12].

1.1. Blood zinc levels

The serum/plasma levels of zinc in AD patients have been reported to be significantly elevated [13], unchanged [14, 15] and significantly decreased [16-19] relative to matched cohorts of healthy patients when assessed using a variety of methods such as inductively coupled plasma mass spectrometry and atomic absorption spectrophotometry.

While the cause of the diversity in these data is not known, there are a number of factors that could contribute to the variation between studies. Gonzalez and colleagues [20] for example, examined serum zinc levels in a cohort of 51 AD subjects ($n=34$ ApoE4 positive) and 40 controls ($n=9$ ApoE4 positive) and found no difference in serum zinc levels between groups when examining all subjects in the cohort. However, when stratified by ApoE4 genotype there was a significant increase in serum zinc levels in the AD group. Thus, if a study cohort is biased with a variable number of E4 allele carriers, this may affect the outcomes of any investigations into serum zinc content.

Another potential confound in these studies is the classification of the diagnostic groups. Dong and colleagues [21], for example, examined serum zinc levels in 18 mild to moderate AD, 19 mild-cognitive impairment (MCI; MCI patients have a 10-15% conversion rate to AD/year with a total of ~80% converting within six years of follow up) and 16 age-matched controls. When comparing all subjects there was no difference in serum zinc levels between groups. However, when segregated by gender a significantly lower level of serum zinc was revealed in male MCI subjects when compared to both female MCI subjects and healthy control male subjects. Thus, there is the possibility that serum zinc levels may vary as a function of disease state.

The other peripheral compartment that has been studied for zinc levels is the cerebrospinal fluid (CSF).

1.2. CSF zinc levels

Initial studies by Hershey and colleagues [22] found no difference in CSF levels of zinc between 33 cases of dementia of the Alzheimer type and 20 non-age-matched cases without neurological disease (these cohorts included 7 AD cases and 8 controls, as verified at autopsy). This finding has been confirmed in a more recent study [23] that examined a cohort of patients with dementia of the Alzheimer's type (n=34) and healthy controls (n=34, aged 40-80 years).

In contrast, Molina and colleagues [14] examined AD (n=26) and control (n=28) patients and found a significant decrease in CSF zinc levels in the AD cohort. Consistent with this, Kapaki and colleagues [24] found a similar effect in a smaller cohort of patients (n=5 AD and n=28 non-age-matched controls).

These CSF reports clearly lack the rigor of the blood studies, as there are far fewer reports and those that have been conducted are relatively under-powered and often lack the most appropriate age-matched controls. Both the blood and CSF studies also suffer from a difficulty in interpreting the relevance of changes in zinc in these peripheral compartments to the pathogenesis of AD. In contrast, the studies of cerebral zinc levels are more tangible in implicating zinc homeostasis in AD.

1.3. Brain zinc levels

Bulk tissue zinc levels have been analysed in different regions of the AD brain with varying results.

The earliest reports suggested that there was no difference in brain zinc levels between AD and controls [25, 26]. This was later confirmed using instrumental neutron activation analysis of frontal lobe tissue, where no difference in whole-tissue samples was seen between AD and control. However, when these tissues were subfractionated a significant decrease in zinc levels in the nuclear fraction (but not the mitochondrial or microsome fraction) of AD cases was found [27]. Similar findings of decreased zinc levels in AD tissue have also been found in the neocortex [28] and in the superior frontal and parietal gyri, the medial temporal gyrus and thalamus [29] and the hippocampus [29, 30]. In the study by Panayi and colleagues [29] tissue from both hemispheres was analysed, revealing the same decreased zinc levels in all regions.

In contrast, a number of studies report that zinc levels are in fact increased in the AD brain, as compared to healthy age-matched controls.

Elevated levels of zinc have been found in the amygdala [28, 31-33], hippocampus [28, 33], cerebellum [28], olfactory areas (including olfactory bulb, olfactory tract, olfactory trigone) [32] and superior temporal gyrus [34], although a combined analysis of the superior and middle temporal gyri did not reveal a change in zinc [33].

There remains, therefore, considerable controversy as to the zinc status of the AD brain. This may, in part, be due to the methods used for the preparation of the tissues, as fixation has been shown to significantly decrease brain zinc levels/detection [35]. One study has indicated that elevated zinc levels were only evident in advanced disease, when plaque burden becomes conspicuous [34]. More recent studies have taken a more spatially detailed approach to the assessment of cerebral zinc levels by examining zinc load specifically within plaques of affected brain areas.

1.4. Plaque and neuropil zinc levels

A microparticle-induced x-ray emission analysis of zinc load within the plaques and surrounding neuropil in the cortical and accessory basal nuclei of the amygdala was undertaken by Lovell and colleagues [36]. These studies revealed a two to three fold increase in zinc levels in the neuropil of AD patients, consistent with bulk-tissue reports, as compared to controls. In addition, zinc levels were even further elevated within the plaques, with a three to four fold increase above that seen in the normal age-matched neuropil. These data are consistent with other histochemical ([37]; hippocampal, amygdalar and neocortical tissue), autometallographic tracing ([38]; hippocampal and neocortical tissue) and synchrotron-based infrared and X-ray imaging ([39]; hippocampal and “frontal” tissue) analyses that have clearly demonstrated a focal accumulation of zinc within the plaques present in the AD brain.

Raman microscopy has demonstrated that senile plaque cores isolated from the cortical grey matter of AD patients consist primarily of the β -amyloid ($A\beta$) protein (derived from a larger trans-membrane protein, coined the amyloid precursor protein (APP)) with both copper and zinc coordinated to histidine residues located at the N-terminal end of the protein [40] (metal binding to synthetic $A\beta$ is mediated by histidine residues at positions 6, 13 and 14). Thus, $A\beta$ (and also APP) is in fact a metalloprotein that directly binds metals such as zinc, and whose function can be modulated by alterations in metal ion homeostasis and subsequent binding to the protein. This will be discussed later in this chapter. Such metal binding may, in part, account for the apparent focal accumulation of zinc within the plaque as shown by the various imaging techniques described above.

While the ultimate cause of this apparent zinc imbalance in the AD brain remains unknown, it is likely that a dysregulation in various metal storage and transport proteins within the AD brain may contribute to a failure in zinc homeostasis. With regards to zinc, there are several key protein families involved in the regulation of cellular levels of this metal. These include the Zrt-, Irt-like Protein (ZIP), zinc transporter (ZnT) and metallothionein proteins (see chapters 4 and 8). A number of these proteins show brain region-specific alterations (both increases and decreases) in mild cognitive impairment and preclinical, early- and late-stage AD [41-46]. Furthermore, a number of these proteins, such as the ZnT family, are found not only in the neuropil but also within the plaques themselves [47]. The role of these proteins is extensively reviewed elsewhere [48], and specific proteins, such as ZnT-3, will be discussed later in this chapter.

In knowing that there is a dyshomeostasis and a mis-compartmentalisation of zinc in the AD, we will now examine the potential down-stream consequences of this, with a specific focus on AD-related pathology and symptoms.

2. The Role of Zinc in the Metabolism of $A\beta$ and Tau

2.1. APP and $A\beta$

As noted earlier, APP gives rise to the $A\beta$ protein that is the primary constituent of the plaques found in the AD brain. APP is ubiquitously expressed and has a number of potential functions and interactions [49]. One proposed function, given the existence of specific binding sites within its amino-terminal ectodomain for metals such as zinc [50-

54], is the maintenance of metal ion homeostasis. Zinc binds to a region between position 170 and 188 of APP [50, 51], which is mediated by two key Cys residues (Cys-186 and Cys-187), together with other potential ligands such as Cys-174 and Met-170 [55]. A disturbance in the normal expression of APP might, therefore, result in a dysregulation of metal ion homeostasis within the brain. Another proposed function of APP, which may also be affected by a dyshomeostasis in zinc levels, is in cellular adhesion and neurite outgrowth [53]. More recently we have reported on a novel function of APP, both full-length and soluble species, as an iron-export ferroxidase [56]. In post-mortem AD tissue this activity is specifically inhibited by zinc, suggesting that a disturbance in zinc ion homeostasis may contribute to deficits in ferroxidase activity and subsequent iron accumulation.

The processing of APP to generate varying length A β peptides involves the coordinated activities of a number of different secretases [57-59], all of which are known to have an interaction with different metal species (for review, see [60]). Briefly, the activity of the candidate alpha-secretases TACE (or ADAM-17) and ADAM-10 can both be specifically modulated by zinc. In the case of TACE, its activity can be regulated, at least in part, by a "cysteine-switch" motif that maintains the enzyme in an inactive state via a bond between a cysteine in its prodomain and a zinc atom in its catalytic domain [61-63]. Likewise, the activity of ADAM-10 can be ablated by a mutation in its zinc binding site [64]. Gamma-secretase activity can also be modulated by zinc – specifically by an interaction with one subunit of gamma secretase, presenilin. Zinc has been shown to enhance the synthesis of presenilin 1 [65] and to subsequently increase the generation of its C-terminal fragments. Furthermore, zinc can inhibit the cleavage activity of gamma secretase on various substrates, as assessed *in vitro*, and may thus affect normal A β processing *in vivo* [66].

Following the generation of A β , it too is capable of binding metals (such as zinc) at a number of sites on the protein, with the obligatory zinc binding site mapped to the region 6-28 of A β [50-53]. As noted above, there are a number of histidine residues (at positions 6, 13, 14) that are crucial for this interaction to occur [67-69] and the binding of zinc to the histidine at position 13 is essential for the aggregation of A β to occur [67]. The precise mechanism underlying this facilitation of aggregation has yet to be determined, but it has been suggested that the zinc found in Zn-A β oligomers is able to coordinate both intra- and intermolecularly to bridge two peptides and further, the coordination of zinc reduces the solvation energy for large Zn-A β oligomers to thereby increase their tendency to aggregate [70]. Each of the three histidines is also able to independently coordinate zinc, giving A β the potential to bind three zinc ions simultaneously [71]. There are also other likely ligands for zinc binding, including Asp1 and Glu11, together with less likely ligands such as Asp7 and Tyr10 (for review, see [72]). This binding occurs at physiological pH [73] and the affinity for zinc is in the low micromolar range (1-20 μ M) [52, 74, 75]. In the absence of the His13 residue, as occurs in the rodent A β sequence for example [76], then the metal-induced aggregation of A β [68] is attenuated [52]. The importance of metals in the aggregation of A β has been simply demonstrated in two separate experiments. Firstly, the *in vitro* fibrillisation of A β can be prevented by the strict exclusion of metals from the buffers used for this reaction [77]. Secondly, the treatment of post-mortem AD brains with chelators results in the solubilisation of A β [73]. It is clear then, that metals can promote the A β aggregation pathway.

Another important consequence of the binding of zinc to A β is that it obscures the proteolytic cleavage site [53], thereby inhibiting its ability to be degraded by matrix

metalloproteases (MMP) such as MMP-2 [78]. Removal of the zinc with clioquinol (discussed later in this chapter), however, restored the sensitivity of A β to degradation by MMPs – demonstrating that zinc-induced aggregates of A β are particularly resistant to the normal cellular metabolism.

While much of the discussion has favoured a potentially deleterious effect arising from the binding of zinc to APP and A β , there are several studies that suggest a potentially protective effect of zinc in certain contexts.

Specifically, very low concentrations of zinc may be protective, in stark contrast to the effects seen at higher physiological concentrations. Lovell and colleagues [79] reported that A β /Zn ratios of 1:0.1 and 1:0.01 result in a protective effect against A β toxicity *in vitro* that is mediated, at least in part, by a modulation of Na $^+$ /K $^+$ ATPase activity that prevents the typical calcium dyshomeostasis and cell death that is associated with A β toxicity (the opposite effect was seen at equi-stoichiometric or higher concentrations of zinc).

Garai and colleagues [80] suggested that, *in vitro*, very low concentrations of zinc may eliminate oligomeric A β – thereby limiting the toxicity mediated by soluble oligomers. The physiological relevance of this, however, has yet to be determined. It has also been suggested that zinc-A β aggregates may form so as to inhibit the reduction of Cu $^{2+}$ and the production of hydrogen peroxide that arises from the A β -Cu interaction [81]. In this scenario, then, zinc binding to A β would represent an antioxidant response by displacing pro-oxidant Cu $^{2+}$.

Further consequences of zinc binding to A β include an increased resistance to its proteolytic cleavage at the secretase site, which would subsequently alter the normal metabolism of A β [53]. In addition, zinc binding to A β may increase its adhesiveness and promote the binding of A β to extracellular matrix components such as laminin and fibronectin to subsequently modulate cell adhesion [53].

Zinc-induced aggregates of truncated A β peptides (A β 10-21) have also been shown to have very specific blocking effects on voltage-gated potassium channels in hippocampal CA1 pyramidal neurons [82].

There are, therefore, a multitude of potential consequences that arise from the interaction between zinc and APP or A β . While most research has favoured the study of the amyloid pathway, zinc is found within tangle-bearing neurons [37]. Next we review literature suggesting that zinc may affect various aspects of tau metabolism.

2.2. Tau and other cytoskeletal proteins

There have been several initial studies that have shown a direct binding between metals and the tau protein, possibly favouring a Cu $^{2+}$:tau interaction [83-85]. A more recent report, however, suggests that zinc may also bind tau [86]. In their study, Mo and colleagues [86] used isothermal titration calorimetry to demonstrate that zinc coordinates to monomeric tau via bridging between Cys-291 and Cys-322 as well as two histidine residues. This interaction occurs with moderate (micromolar) affinity and may contribute to tau pathology, with low concentrations of tau shown to induce tau fibril formation and higher concentrations shown to induce the aggregation of the protein [86]. The concentrations utilised in this study were also well below those found in neurons, suggesting relevance to the pathogenesis of AD.

In addition to this direct interaction, zinc may impact a multitude of other cellular pathways, many of which have been reviewed within this book, which may then influence the metabolism of tau. Utilising *in vitro* models, An and colleagues [87] and

Pei and colleagues [88] have shown that zinc may affect both the translation and phosphorylation of tau, the latter via activities on various kinases such as glycogen synthase kinase (GSK)-3 β , protein kinase B, extracellular signal-regulated kinase and c-Jun N-terminal kinase. More recently, Boom and colleagues [89] have demonstrated that varying concentrations of zinc can both dephosphorylate the pathological PHF-1 epitope and induce the formation of the conformational tau epitope MC1, in addition to increasing the activity of GSK-3 β and displacing tau from microtubules. Their data support a role for zinc in modifying the phosphorylation and conformational status of tau that may contribute to the early events leading to the formation of neurofibrillary tangles.

While tau is the primary constituent of the neurofibrillary tangle, there are other cytoskeletal proteins that are known to bind, and be modulated by, zinc. These include neurofilaments and tubulin. Neurofilaments, which are found within neurofibrillary tangles and dystrophic neurites surrounding plaques in the AD brain [90], can bind four moles of zinc [91] while tubulin, whose polymerisation to form microtubules is mediated by zinc [92-94], has multiple binding sites for zinc [95, 96]. It is unclear whether the modulatory effects of zinc on these other proteins is of relevance to the pathogenesis of AD.

3. The Effect of Zinc on Other AD-Related Pathways

In addition to the proteins/pathways already discussed, there are several others that interact with zinc that are also relevant to AD, such as nerve growth factor, Cu-Zn superoxide dismutase and protein kinases (for review, see [97]).

One of the primary protein families of interest, however, are the metallothioneins (MTs). MTs bind zinc, copper and other transition metal ions (7-12 ions/MT), regulate the availability of metal ions for various enzymes and transcription factors, and help regulate the redox status of the cell. In AD, there are various reported studies showing increases, decreases and no change in MT species in the brain. MT-I/II has been reported to be increased in the AD brain [44, 98], while MT-III (also known as growth inhibitory factor for its ability to suppress neuronal growth) has been reported to be both unchanged [99] and decreased in the AD brain [45, 46]. While a dysregulation in the various MT isoforms may have more generalised consequences for the cell, more recent studies suggest a specific role in the pathogenesis of AD. Specifically, it has been reported that Zn₇MT-III may remove copper from both soluble A β 1-40-Cu(II) and aggregated A β 1-40-Cu(II) [100]. One of the consequences of this metal-swap was a quenching of hydroxy radical production by A β 1-40-Cu(II), resulting from the redox cycling of copper in the presence of ascorbate, which in turn prevented A β 1-40-Cu(II)-induced toxicity in SH-SY5Y cells. Thus, MT-III may play a key role in the AD brain and the reported down-regulation of MT-III may thus propagate the disease process.

Another zinc-modulated protein that is emerging as having potential relevance to AD is calcineurin. While early studies did not report any difference in calcineurin immunoreactivity between AD and control subjects [101], more recent reports have linked calcineurin activation to A β -mediated neurodegeneration in AD [102, 103]. Moreover, there is an apparent alteration in calcineurin signalling associated with cognitive decline in AD [104] and calcineurin has also been identified in a SNP analysis as a genetic factor that was strongly associated with the rate of decline in AD [105].

Thus, there are a host of other potential pathways that may be mediated by zinc and which may be of relevance to the pathogenesis of AD.

4. Zinc as a Potential Therapeutic Target for Alzheimer's Disease

There are a number of animal and human studies that have linked a dyshomeostasis in brain metal levels to the initiation/propagation of AD. While several of these have specifically focused on the role of zinc, there are also others, particularly drug intervention trials, that are more broad with regards to which metal they target. In any case, these studies provide important insight into the potential role of zinc in AD.

To examine the influence of synaptic zinc on amyloid pathology, Lee and colleagues [106] crossed Tg2576 mice, which over-express a mutant human form of APP and develop cerebral A β plaques [107], with ZnT3 KO mice, which lack vesicular zinc in glutamatergic synapses [108]. The resulting double mutant mice (ZnT3^{-/-}:Tg2576) had significant reductions in cortical and hippocampal plaques, and the plaques that were present were significantly reduced in size. In addition, the assessment of A β load in whole hemisphere homogenates revealed a significant reduction in insoluble A β species, but a significant elevation in soluble A β species, in the double mutant mice. The ZnT3^{-/-}:Tg2576 animals had a 1.3-1.5 fold higher soluble/insoluble ratios of A β 40 and A β 42, relative to the ZnT3^{+/+}:Tg2576 mice. In these studies it was also reported that the gender bias, of female Tg2576 mice having more plaques than male animals (paralleling the increased synaptic vesicle zinc levels in female mice), was eliminated following the cross with the ZnT3 KO animals. It was subsequently shown that estrogen is able to regulate ZnT3 levels, and thus also synaptic vesicular zinc levels, through an action on the adaptor protein complex, (AP)-3 [109]. These data suggest that zinc may hold amyloid load in a dissociable equilibrium and clearly provide strong support for a role of synaptic zinc in the metabolism of A β . A follow-up study also revealed a reduction in cerebrovascular amyloid levels in these animals, with ZnT3 shown to promote cerebral amyloid angiopathy by elevating exchangeable Zn²⁺ levels in the perivascular spaces of vessel walls [110].

In contrast, when TgCRND8 and Tg2576 mice were chronically supplemented with 10ppm zinc carbonate in their drinking water, there was a significant reduction in the immunohistochemical detection of A β plaques [111], parallel with a reduction in plaque diameter. However, despite the improvements in brain pathology in the zinc-treated mice, they were the worst performers in the spatial learning and memory tasks, suggesting that the zinc-enriched diet potentiated the cognitive impairments in these animals. These data are consistent with work from Stoltzenberg and colleagues [112] who demonstrated that APP/PS1 animals raised on a zinc-deficient diet had a significant elevation in cortical plaque area and volume, suggesting that zinc deficiency may influence A β pathology.

More recent studies have suggested that the attraction and subsequent binding of A β oligomers to the zinc released at the glutamatergic synapse may both occlude the NMDAR2B receptor and prevent the trans-synaptic movement of zinc, thus inhibiting any zinc modulatory activity on crucial post-synaptic targets to thereby potentiate cognitive dysfunction in AD [113]. Consistent with this, our own studies [114] suggest that the genetic ablation of ZnT3 may generate a phenocopy for the cognitive and synaptic deficits present in transgenic mouse models of AD.

It is apparent then, from both the *in vitro* and *in vivo* work discussed here, that there is a complex regulation of synaptic zinc in both health and disease and that a dyshomeostasis in zinc could promote AD pathology and cognitive symptoms. To this effect, there are a number of compounds that have been developed that normalize cerebral zinc ion homeostasis.

Lee and colleagues [115], for example, tested a lipophilic metal ion chelator, DP-109, which has a selectivity for zinc and copper and to a lesser extent other metals such as iron [116]. Chronic treatment of aged Tg2576 mice with DP-109 resulted in a significant reduction in A β burden within the brain, and this occurred in parallel with an elimination of vesicular zinc, suggesting a potential mechanism of action of this compound that is consistent with the earlier reports. Other metal-modulatory compounds that have been investigated include clioquinol and PBT2.

Cherny and colleagues [117] were the first to study the effect of clioquinol treatment in aged Tg2576 mice, which resulted in a significant reduction in plaque burden parallel with a shift in brain content of A β towards more soluble species. It is of note that an analysis of cerebral metal levels did not reveal a reduction in metals, but rather, a small but significant increase in copper and zinc upon treatment. These data, and subsequent studies, have revealed that compounds such as clioquinol and PBT2 (discussed later) actually redistribute metals within the brain and do not chelate metals out of the system – although the effect of the compounds may vary depending upon the dose and route of application [118].

The effect of clioquinol in a transgenic mouse model of AD has recently been repeated [119]. This study also confirmed our earlier report on the cognitive benefits of clioquinol in transgenic mouse models of AD [120]. Briefly, Grossi and colleagues demonstrated an improvement in cognition in parallel with the decreased A β burden and elevated metal levels in the parietal cortex and hippocampus of TgCRND8 animals following treatment with clioquinol. The alteration in metal levels was consistent with the localisation of clioquinol, as assessed with MALDI-TOF.

We have recently demonstrated that a more potent successor to clioquinol, PBT2, has profound effects in transgenic mouse models of AD [120]. Utilising both Tg2576 and APP/PS1 animals, we demonstrated that brief (11 day) administration of PBT2 resulted in a significant decrease in both insoluble *and* soluble A β burden, decreased levels of phosphorylated tau, increased levels of synaptophysin (a surrogate marker for synapses) and rapidly improved learning and memory performance in the Morris water maze. The cognitive effects were rapid, occurring within as little as six days after exposure to the drug, consistent with the rapid effects of PBT2 on interstitial fluid A β levels, which were seen within hours after a single oral dose. The strong preclinical data package for PBT2 resulted in it proceeding successfully through a phase IIa human clinical trial, where there was a significant decrease in CSF A β 42 levels, as well as a significant improvement in executive function tasks [121, 122].

5. Conclusion and Perspectives

It is clear that metal ion homeostasis may be a central player in the initiation and propagation of the pathological features and symptomatic progression of AD. This notion is clearly annunciated in the wealth of *in vitro* and *in vivo* preclinical data that has emerged over the last decade. While there is conflicting biochemical data in the clinical literature (perhaps reflecting the complexity of the human metallome in

degenerative disorders such as AD), the therapeutic studies that target this failure in metal ion homeostasis will provide the burden of proof as to the voracity of this target in AD. To this end it is becoming apparent that "classical" approaches to the treatment of AD are failing to meet expectations, with the recent failure of several anti-A β therapeutic agents. This has led to the suggestion that such approaches may only be efficacious in pre-symptomatic individuals and that more holistic approaches are required. Approaches targeted at metal ion homeostasis, which effect multiple components in the spectrum of AD pathology, may thus fill this niche and prove to be the first disease-modifying strategy for the treatment of AD.

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22. Zinc and Stroke

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Abstract. Over the past three decades, we have learned much about the pathophysiology of brain ischemic injury. Much of this work focused on effects initiated by rapid release of the excitatory neurotransmitter, glutamate, and downstream Ca^{2+} overload. Yet therapeutic interventions based upon these mechanisms have had limited success. More recently, a second divalent cation, Zn^{2+} , has garnered considerable attention as a signal ion and mediator of damage in brain ischemia. Zn^{2+} accumulates in damaged neurons after ischemia in many areas of the mammalian forebrain and contributes in diverse ways to the injury process. The past decade has seen rapid advances in our understanding of ways in which Zn^{2+} may contribute to distinct stages of the ischemic neurodegeneration cascade. It is hoped that this emerging understanding will suggest new and more effective treatments to decrease morbidity after cerebral ischemia.

Keywords. Zn^{2+} Ischemia, Calcium, Mitochondria, Glutamate, Cell death, ROS

Introduction

Brain ischemia, generally occurring in the context of stroke or cardiac arrest, is a leading cause of human morbidity and mortality. However, treatment is presently limited largely to prevention, or to restoration of blood flow within a relatively short time window, before irreversible injury occurs. Considerable study over several decades has been focused on the basis for the extraordinary sensitivity of brain to loss of blood flow, with the hope that neuroprotective strategies could be developed that would extend the time window for restoration of perfusion without injury, or diminish delayed injury after transient ischemia. One critical clue was that the excitatory neurotransmitter, glutamate, is released in large quantities during ischemia [1] and that the consequent activation of postsynaptic N-methyl-D-aspartate (NMDA) type glutamate receptors permit rapid and injurious Ca^{2+} entry. Indeed, a number of studies showed a clear relationship between sharp rises in intraneuronal Ca^{2+} levels (termed “ Ca^{2+} deregulation”) and cell death [2-4]. However, despite some promise in some animal models, extensive human trials, largely with NMDA receptor blockers, repeatedly failed to demonstrate efficacy [5]. Interestingly, the ongoing search for factors that might play critical roles in the ischemic neurodegeneration cascade has led to growing interest in another divalent cation, Zn^{2+} .

Zn^{2+} is present in synaptic vesicles of many excitatory pathways in the mammalian forebrain, from which it can be co-released with glutamate upon presynaptic activation. The observation that after ischemia, Zn^{2+} appeared to be lost from presynaptic terminals, and to accumulate in degenerating postsynaptic neurons led to the idea that

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synaptic Zn²⁺ release and its "translocation" into post synaptic neurons might contribute critically to neurodegeneration [6], an idea give a further boost by findings that an extracellular Zn²⁺ chelator, Ca-EDTA decreased both the Zn²⁺ accumulation and injury after ischemia [7, 8]. (As discussed below, whereas new clues to actions of Zn²⁺ in ischemia are rapidly accumulating, the above description of Zn²⁺ translocation appears to be a gross oversimplification of actual Zn²⁺ dynamics in ischemia).

It is hoped that the emerging understanding of Zn²⁺ mechanisms will result in new and effective neuroprotective interventions to decrease morbidity from cerebral ischemia. This chapter aims to outline ways in which Zn²⁺ ions, derived from multiple sources, may participate in distinct stages of ischemic injury cascades and contribute along with Ca²⁺ to neuronal death.

1. Sources of Intracellular Zn²⁺ Accumulation

1.1. Synaptic Vesicular Release

Timm's sulfide silver staining of the hippocampal formation yields a familiar horseshoe shaped pattern, due to labeling of the dentate granule cells and their mossy fiber projections to CA3 pyramidal neurons. This distinctive labeling pattern reflects Zn²⁺ sequestered within presynaptic vesicles of many excitatory (glutamatergic) terminals [9]. However, although the mossy fibers constitute the densest Zn²⁺ containing pathway, presynaptic vesicular Zn²⁺ is present in many pathways throughout the mammalian forebrain, and its effects are widespread [10].

Although stimulation evoked Zn²⁺ accumulation in the extracellular space was first demonstrated more than 2 decades ago [11-16] and microdialysis studies have demonstrated its extracellular accumulation during brain ischemia [17, 18], there has been considerable uncertainty and controversy over the amounts of Zn²⁺ released and likely peak levels attained in the synaptic space [19]. The uncertainty has reflected a number of factors, including the high affinity of many of the fluorescent indicators employed, and limitations in the spatial and temporal resolutions of many of the approaches used. The production of transgenic mice lacking the vesicular Zn²⁺ transporter, ZnT3, which have no presynaptic vesicular Zn²⁺ (and correspondingly lack the distinctive Timm's staining pattern in the dentate gyrus and mossy fiber pathway) provided a valuable tool to assess activity dependent release of presynaptic Zn²⁺ and its effects [20]. A recent study demonstrating presynaptic stimuli to induce discrete rises in Zn²⁺ in the extracellular space, which were absent in ZnT3 knockout mice and were modified by manipulations that also modify synaptic glutamate release, provided strong confirmation of the presumption that presynaptic Zn²⁺ is co-released with glutamate [14].

Once Zn²⁺ is released into the synapse, it could potentially act at a range of extracellular receptor sites to modify neuronal injury in conditions like ischemia. Some of these extracellular Zn²⁺ actions may be neuroprotective, such as the blockade of postsynaptic NMDA type glutamate channels [21-24], or possible stimulation of presynaptic K_{ATP} current, resulting in decreased presynaptic release [25, 26]. Other extracellular effects of Zn²⁺ could act to increase injury, including the potentiation of AMPA type glutamate receptor mediated currents [21, 27, 28], or blockade of extrasynaptic GABA mediated inhibitory currents (possibly after strong release and synaptic spillover) [29-31]. Zn²⁺ could also activate a g-protein coupled receptor ("Zn²⁺

sensing receptor”), resulting in downstream signaling including intracellular Ca^{2+} mobilization, with complex effects on injury [32].

In addition, after entry into the synapse, Zn^{2+} could enhance injury via entry into postsynaptic neurons through certain routes. Of primary interest are channels that can be directly activated in response to glutamate. Although Zn^{2+} is an effective blocker of NMDA type glutamate channels, studies in culture revealed that small amounts can permeate these channels and mediate toxicity [33]. In addition, under conditions of neuronal depolarization, Zn^{2+} can permeate voltage gated Ca^{2+} channels (VGCC), mediating toxicity after entry through that route [34-36]. In addition, whereas most AMPA type glutamate channels mediate routine excitatory neurotransmission via Na^+ conductances and are impermeable to Zn^{2+} , some AMPA channels, which lack the GluR2 subunit are Ca^{2+} permeable (Ca-AMPA channels). These channels are also highly Zn^{2+} permeable [37-39]. Culture studies suggest a rank order of permeability, Ca-AMPA > VGCC >> NMDA channels [38]. Whereas Ca-AMPA channels are strongly expressed on many forebrain inhibitory interneurons, they are not generally felt to be prevalent on most pyramidal neurons under basal conditions, although a number of studies suggest that there are some of these channels present particularly in dendrites at a distance from the soma where they may be relatively hard to detect by electrophysiological recording [40-42]. Furthermore, there is evidence that Zn^{2+} entry through these channels contributes to ischemic injury of HPNs, both reflecting acute entry [43], and perhaps more importantly to delayed injury, after downregulation of the GluR2 subunit (and corresponding upregulation of these channels) occurring 1-2 days after transient ischemia [44]. Other Zn^{2+} entry pathways relevant during ischemia may include exchange with Na^+ [36, 45], and possibly ZIP family Zn^{2+} transporters [46].

1.2. Liberation from Intracellular Stores

In light of the presence of presynaptic vesicular Zn^{2+} , and its release during ischemia accompanied by its accumulation in degenerating postsynaptic neurons, it was widely assumed that Zn^{2+} translocation was an important contributor to injurious intracellular Zn^{2+} accumulation in ischemia. Thus the observation that in ZnT3 knockout mice, which lack presynaptic vesicular Zn^{2+} , induction of seizures actually resulted in greater Zn^{2+} accumulation in CA1 pyramidal neurons than was seen in wild type animals [47] was initially greeted with surprise.

However, it has been long known that cells contain major pools of protein bound Zn^{2+} distinct from vesicular Zn^{2+} . Indeed, Zn^{2+} is an essential cofactor for many enzymes and transcription factors, and its levels throughout neurons are tightly regulated by the combined actions of transporter proteins and intracellular binding proteins. Metallothioneins (MTs) constitute a major family of Zn^{2+} binding proteins (see chapter 4). They have 20 cysteine residues, the thiol groups of which can coordinate 7 Zn^{2+} ions with differing affinities. Under conditions of oxidative stress, the thiols can be oxidized to disulfides with displacement of Zn^{2+} . Thus, although MT itself is redox inactive, its binding to MTs (and consequently its cytosolic levels) is highly redox sensitive and is destabilized by oxidants including nitric oxide (NO) and by acid pH [48-50]. In neurons, the major metallothionein isoform is MTIII.

Interestingly, consistent with the idea that intracellular Zn^{2+} mobilization contributes to neurodegeneration *in vivo*, exposure of cultured neurons to a disulfide oxidant caused intracellular Zn^{2+} mobilization that contributed to their injury [51]. Specifically implicating MTIII as a major source of intracellular Zn^{2+} in CA1

pyramidal neurons, knockout of MTIII decreased seizure induced Zn²⁺ accumulation and neuronal death [52]. However, MTIII does not only act as a source of oxidant released Zn²⁺, but can also buffer cytosolic Zn²⁺ rises caused by rapid influx into cells [53]. Indeed, in contrast to CA1, in CA3 pyramidal neurons, MTIII removal worsened seizure induced injury [52, 54], and elimination of synaptic Zn²⁺ (in ZnT3 knockouts) decreased injury [47], suggesting that in these neurons, upon which the mossy fibers provide a particularly dense Zn²⁺ containing input, release of vesicular Zn²⁺ and its entry into postsynaptic neurons contributes strongly to injury, and buffering of Zn²⁺ rises by post synaptic MTIII is beneficial. Similarly, MTIII knockout was recently reported to result in increased injury in a focal cerebral ischemia model [55].

In conclusion it appears that there are two pools of Zn²⁺, presynaptic vesicular and post synaptic protein bound, either of which has the potential to contribute to injurious intracellular accumulation in ischemia, but that the specific contributions are complex and likely vary with cell type and the nature of the insult. Thus, whereas synaptic Zn²⁺ release and its translocation into the postsynaptic neuron may in some cases contribute to ischemic injury, removal of presynaptic Zn²⁺ may also exacerbate injury, perhaps by permitting increased activation of NMDA type glutamate receptors. And conversely, whereas buffering of Zn²⁺ entry by MTIII may protect, it also appears that release of Zn²⁺ from MTIII under conditions of oxidative / nitrosative stress and acidosis can contribute to neuronal injury.

2. Cellular Mechanisms of Zn²⁺ Neurotoxicity

Studies, largely carried out in simplified *in vitro* systems, have highlighted numerous mechanisms through which Zn²⁺ may contribute to neurodegeneration in ischemia. A common feature of many of the implicated mechanisms is alteration of metabolic pathways, often with evidence of increased oxidative stress.

2.1 *Multiple and Potent Effects of Zn²⁺ on Mitochondrial Function*

A number of studies outlined below highlight mitochondria as one locus of important Zn²⁺ effects. Zn²⁺ appears to induce effects on mitochondria following its entry through the Ca²⁺ uniporter, and possibly other routes [56-59]. Interestingly, past studies indicating that Zn²⁺ binding to metallothioneins is highly redox sensitive, combined with findings that MTs can carry Zn²⁺ into mitochondria, inhibiting respiration proportionately to the amount of Zn²⁺ carried [50, 60] suggest the possibility that Zn²⁺ binding by MT's and mitochondrial Zn²⁺ accumulation may subserve physiological functions in the autoregulation of mitochondrial metabolism.

Studies have indicated that Zn²⁺ can affect mitochondrial function in a number of ways. Functionally, Zn²⁺ has been found to induce mitochondrial depolarization and swelling both in isolated mitochondria and in intact neurons. This effect is likely due in large part to activation to the mitochondrial permeability transition pore (mPTP) or a similar large conductance channel [56, 57, 61-63]. Zn²⁺ also induces reactive oxygen species (ROS) generation and/or release from mitochondria [38, 57, 64, 65]. Highlighting the potency of Zn²⁺ effects on mitochondria, we found 10 nM Zn²⁺ to induce swelling of isolated mitochondria comparable to that induced by 100 μM Ca²⁺, which was blocked by the mPTP antagonist bongkrekic acid [57]. These conclusions are not all settled however, as not all find equally potent Zn²⁺ induced mPTP opening

[66], possibly in part reflecting organ differences (liver vs brain), and uncertainty remains regarding both the amount and mechanisms of ROS generation occurring with physiologically relevant Zn²⁺ exposures.

2.2. Other Effects Linked to Metabolic Inhibition

Zn²⁺ appears to have other metabolic effects largely independent of direct effects on mitochondria. In cultured neurons Zn²⁺ has been found to trigger activation of protein kinase C (PKC), resulting in induction and activation of NADPH oxidase, and also to induce nitric oxide synthase (NOS) [67-69]. NADPH oxidase uses NADPH as substrate and produces superoxide. In addition to being a damaging free radical of its own accord, superoxide reacts with NO, the product of NOS, to produce the highly injurious radical peroxynitrite.

Free radicals (like superoxide and peroxynitrite) can cause DNA strand breaks, resulting in activation of the enzyme poly(ADP-ribose) polymerase (PARP), which catalyzes attachment of ADP ribose units from NAD⁺ to various nuclear proteins and plays a role in DNA repair. PARP activation depletes NAD⁺ and ATP, and contributes to ischemic brain injury [70]. Consistent with activation of this pathway, Zn²⁺ has been specifically shown to cause PARP activation and NAD⁺ depletion, and blockers of NADPH-oxidase, NOS or PARP attenuated delayed Zn²⁺ induced degeneration of cultured neurons [67].

Interestingly, the NAD⁺ depletion caused by PARP in response to Zn²⁺ or to excitotoxic NMDA receptor activation, rather than direct PARP dependent depletion of ATP may be integral to the downstream injury pathway. PARP activation has been found to lead to release of apoptosis-inducing factor (AIF) from the mitochondria, which translocates to the nucleus and triggers caspase independent DNA fragmentation and cell death [71]. Whereas some studies suggested that PAR (the polymer product of PARP) can directly induce AIF release from mitochondria [72], a distinct and alternate mechanism was provided by the finding that NAD⁺ depletion per se results in a block of glycolysis, with consequent loss of substrate flow into mitochondria and release of AIF [73]. Pyruvate is highly protective against Zn²⁺ neurotoxicity (as well as ischemic injury) [74, 75], perhaps because it can bypass the glycolytic block, while promoting regeneration of NAD⁺ via the enzyme LDH [73, 75]. Other mechanisms likely also contribute to Zn²⁺ dependent NAD⁺ depletion. Sirtuins are NAD⁺ catabolic enzymes, activators of which enhanced NAD⁺ loss and Zn²⁺ neurotoxicity, and antagonists of which were protective [76].

2.3. Other Signalling Pathways: MAP Kinases etc.

Mitogen-activated protein (MAP) kinases are critical signaling pathways in the regulation of cellular survival, proliferation and death (see chapter 5 and 6). The family comprises c-Jun N-terminal kinases (JNK), extracellular signal-regulated kinases (ERK) and P38 MAP kinases, each of which is regulated by a cascade of phosphorylation events. Phosphorylated MAPKs either interact with cytosolic substrates or translocate to the nucleus and modulate transcription factors. In many cases these pathways are activated in response to cellular stressors and they have been found to be strongly activated in conditions including ischemia. Despite their complex and in some cases survival associated roles, recent studies have implicated activation of P38 and ERK MAP kinases in distinct Zn²⁺ induced neurodegeneration pathways.

In neuronal cultures exposed to oxidants [2,2'-dithiodipyridine (DTDP) or NO], intracellular Zn²⁺ mobilization (likely upon release from MT's or other redox sensitive binding sites) has been found to result in activation of p38 MAP kinase [51, 77, 78], which contributes to the phosphorylation and membrane insertion of the Kv2.1 K channels [79, 80], increased K⁺ currents, caspase 3 and 9 cleavage and cell death. In this paradigm, the precise upstream determinant of p38 activation is uncertain, but may involve a combination of oxidative stress, PARP activation, and mitochondrial dysfunction [77, 78, 81], all of which could be triggered by Zn²⁺ interaction with mitochondria and/or NADPH oxidase induction.

Whereas ERK1/2 activation has most often been associated with cell survival and differentiation, a number of studies have found that, with prolonged activation, it can trigger cell death. ERK1/2 activation has been most often (but not exclusively) reported to contribute to Zn²⁺ induced neuronal cell death after brief exposures of neurons to extracellular Zn²⁺ (in contrast to the oxidant induced intracellular Zn²⁺ mobilization most closely implicated in P38 MAP kinase dependent injury) [82, 83]. In this pathway, inhibition of phosphatases by Zn²⁺ may contribute to ERK1/2 activation, which appears to be upstream of mitochondrial dysfunction, ROS generation, NADPH oxidase, PARP activation, activation of transcription factors like Egr-1 or Elk-1, and caspase independent cell death. Other studies suggest that Zn²⁺ rises trigger 12-lipoxygenase activation, causing ROS generation and contributing to P38 MAPK and ERK1/2 activation [81, 84].

2.4. Zn²⁺ Dependent Modification of AMPA Receptor Subunit Composition

One particularly intriguing mechanism through which Zn²⁺ may play a role in delayed neurodegeneration, up to several days after a transient ischemic insult, concerns the modulation of a likely major route of synaptic Zn²⁺ entry. Specifically, as discussed above, Ca-AMPA channels are highly permeable to Zn²⁺ and are generally felt to be either absent or present at relatively low levels in most adult hippocampal pyramidal neurons under basal conditions. The Ca²⁺ permeability of the channels is regulated by GluR2 AMPA subunits, the presence of which in heteromeric channels prevents their permeability to Ca²⁺. Transient ischemia has been found to cause a delayed decrease in GluR2 mRNA in hippocampal pyramidal neurons and to result in a greater proportion of AMPA channels lacking this subunit, which are permeable to Zn²⁺ as well as to Ca²⁺ [44, 85, 86]. Zn²⁺ may contribute to the induction of the signaling pathway leading to the GluR2 downregulation, as it is prevented by the addition of Zn²⁺ chelators close to the time of the transient ischemia [87]. Further studies have found that the downregulation appears to be mediated in part via upregulation of a transcriptional repressor, REST (repressor element-1 silencing transcription factor)/NRSF (neuron-restrictive silencing factor), which represses expression of a subset of genes including GluR2 [88]. Thus, it will be interesting to try to determine mechanisms through which Zn²⁺ rises may lead to REST activation. Interestingly, Zn²⁺ flux through the newly inserted Ca-AMPA channels may contribute to the delayed neurodegeneration 2-3 days after the transient ischemia, as late application (at the time to channel upregulation) of either Zn²⁺ chelators or Ca-AMPA channel blockers attenuate both intracellular Zn²⁺ accumulation and delayed cell death [87, 89].

2.5. Zn²⁺ Effects on Non-Neural Cells

It is clear that the development of ischemic brain injury involves not only neurons but the involvement of other cell types with which they interact. For instance, astrocytes

are the primary neuronal support cells in the brain, and carry out many functions to maintain a healthy milieu for neuronal function and survival. One critical role of astrocytes that comes into play during ischemia is the re-uptake of glutamate from the extracellular space. Loss or reversal of glutamate uptake can lead to prolonged and substantial elevations of extracellular glutamate exacerbating excitotoxic injury. Interestingly, a recent report found that Zn^{2+} could inhibit glutamate uptake in cultured astrocytes via a mechanism involving PARP activation [90].

Another cell type that plays roles later in the injury process are microglia, the resident immune cells of the central nervous system. Under pathological conditions including stroke microglia can become activated and release ROS and inflammatory mediators, which can contribute to neuronal injury hours to days after the initial event. A recent study found that Zn^{2+} could induce microglial activation via a mechanism involving the sequential activation of NADPH oxidase, PARP and NF-kappaB [91].

Thus, release of Zn^{2+} from neurons and its early extracellular accumulation in stroke could set into motion events in other types of cells that may play roles in distinct stages of the injury; inhibition of glutamate uptake by astrocytes could enhance the early excitotoxic component of the injury, whereas microglial activation could contribute to late stage inflammatory components.

2.6. Possible Feed-Forward Amplification of Injurious Signals

The discussions above highlight a number of specific mechanisms and targets through which Zn^{2+} could contribute to ischemic neurodegeneration. However, the pathways are not all distinct, and their interactions may provide opportunity for amplification and acceleration of the injury process.

First, it is likely that there is positive feedback between intracellular Zn^{2+} mobilization and oxidative stress. For instance, metabolic sequelae of ischemia (including ROS generation and acidosis) cause Zn^{2+} release from intracellular protein bound stores (like MTs), and possibly from depolarized mitochondria as well [62, 92, 93]. However, the increased Zn^{2+} mobilization can block metabolic pathways, and may induce ROS generation from mitochondria, NADPH oxidase, NOS and other sources (including 12-lipoxygenase), causing further Zn^{2+} release.

Also, there is evidence for possible cross talk between specific pathways discussed above. For instance, in addition to direct mitochondrial effects, Zn^{2+} may impair mitochondrial function and induce AIF release via NAD^+ depletion. Also, mitochondrial dysfunction may be both upstream of p38 MAP kinase activation and downstream from ERK activation. Interestingly, it has been reported that mitochondrial ROS may induce delayed NADPH oxidase activation [94], and that NADPH-oxidase activation may be able to promote mitochondrial ROS release [95]. If both of these mechanisms occurred it could provide a basis for a feed-forward cascade amplifying oxidative injury. Perhaps Zn^{2+} accumulation, metabolic inhibition and oxidative stress are prominent upstream events which can amplify themselves as well as triggering more slowly evolving injury pathways such as those activated by MAP kinases or alteration in AMPA receptor subunit composition. If the initiating event is sufficiently intense, the cell might rapidly degenerate. However, with sublethal initial events, complex and interacting Zn^{2+} triggered cascades may come into play in the determination of cell fate.

3. What Happens *in vivo*: Clues from Slice Models

3.1. Zn^{2+} vs. Ca^{2+} : which is the Real Culprit?

One of the critical outstanding questions concerning ionic triggering mechanisms in ischemic neurodegeneration concerns the discrimination between effects and roles of Ca^{2+} and Zn^{2+} . This difficulty has reflected a number of factors. First, despite numerous studies demonstrating that neuronal Ca^{2+} overload can trigger lethal neuronal injury, some of the supporting studies carried out in slice models based inferences on use of blockers of Ca^{2+} permeable channels (including VGCC and glutamate channels), Ca^{2+} chelators, and fluorescent Ca^{2+} indicators. However, it is now clear that certain " Ca^{2+} channels", like VGCC and Ca-AMPA channels are quite permeable to Zn^{2+} (see chapter 7), and that virtually all " Ca^{2+} indicators" also respond to Zn^{2+} with higher affinity than Ca^{2+} (see chapter 9). This realization led to studies using Zn^{2+} selective vs. " Ca^{2+} selective" indicators in slices subjected to oxygen glucose deprivation (OGD) as an *in vitro* model of ischemia, and led to the suggestion that Zn^{2+} rather than Ca^{2+} may be the principal instigator of neuronal damage [96]. However, what had been lacking was an effort to simultaneously measure Zn^{2+} and Ca^{2+} in the same cells in order to glean clues to their respective contributions.

A technical issue however, concerns the difficulty in measuring both ions in light of the fact that the available Ca^{2+} indicators are Zn^{2+} sensitive. However, recent studies suggest that the discrimination is possible, in part because of the far greater absolute levels of Ca^{2+} than Zn^{2+} that occur. Specifically, using high affinity Zn^{2+} indicators along with relatively low affinity Ca^{2+} indicators, at concentrations that are far higher than the intracellular Zn^{2+} transients, binding of small amounts of Zn^{2+} have little effect on total fluorescence of the Ca^{2+} indicator, while the far greater Ca^{2+} rises are readily detected [97].

Using this general approach, recent studies have sought to load single pyramidal neurons in hippocampal slice preparations with Zn^{2+} and Ca^{2+} indicators during injury paradigms to discriminate contributions of these ions. In one study, single CA1 neurons were co-loaded with Zn^{2+} and Ca^{2+} sensitive indicators before excitotoxic NMDA exposure. Zn^{2+} rises preceded Ca^{2+} rises, which were in turn closely linked to membrane leakage and necrotic degeneration. If the Zn^{2+} was chelated, the Ca^{2+} rises and cell death were delayed but not prevented [98]. Another study set out to examine the role of Zn^{2+} in the triggering of waves of profound neuronal depolarization, called spreading depressions (SD), that propagate across brain (or slice) tissues after various insults and appear to contribute to the extension of injury after brain ischemia. It has long been apparent that glutamate is involved in the propagation of SD, presumably released from presynaptic terminals that are depolarized during the propagation of the event. In this study, Zn^{2+} accumulation in CA1 neurons, in part after entry through VGCC, preceded and contributed to the initiation of SD events induced by blockade of the plasma membrane Na^+/K^+ ATPase, and Ca^{2+} rises were not seen until the onset of the SD event [99]. In another study, CA1 neurons were co-loaded with Zn^{2+} and Ca^{2+} indicators prior to subjecting the slice to OGD. In this study, as in the excitotoxic exposure, Zn^{2+} rises preceded Ca^{2+} rises, which were in turn closely linked to membrane leakage and cell death. Again, Zn^{2+} chelation delayed but did not prevent the Ca^{2+} rise and cell death [100]. Thus, taken together, these studies seem to indicate that both Zn^{2+} and Ca^{2+} play distinct and important roles in neurodegeneration. The Zn^{2+} appears to accumulate early and contributes to the conditions underlying an abrupt

deregulation of intracellular Ca^{2+} . However, the Ca^{2+} appears to be directly linked to the onset of membrane failure and cellular necrosis, possibly after activation of Ca^{2+} sensitive catabolic enzymes.

3.2. Endogenous Zn^{2+} Accumulation may Mediate Acute Effects on Mitochondria

Most of the studies discussed above concerning Zn^{2+} effects on mitochondria were based on addition of exogenous Zn^{2+} . No doubt some of the exposures were at non-physiological levels, and it is therefore hard to infer the nature of deleterious mitochondrial effects that may occur upon accumulation and mobilization of endogenous Zn^{2+} . Several mechanisms seem likely. One is simple inhibition of energy production. Another is via ROS production/release, which could be due to a number of mechanisms including e-transport inhibition, loss of antioxidant defenses, or by triggering brief openings of the mPTP which may cause release of superoxide puffs [101-103]. In addition, Zn^{2+} activation of the mPTP may trigger mitochondrial release of compounds like cytochrome C or apoptosis-inducing factor (AIF), which can activate apoptotic cell death pathways [57].

Despite uncertainty as to the specific nature of the effects, a number of studies to date do support contributions of endogenous Zn^{2+} to mitochondrial dysfunction in models of ischemia. Zn^{2+} chelation at the onset of ischemia attenuated mitochondrial cytochrome C release and downstream caspase 3 activation [87]. Also, ischemia was found to induce Zn^{2+} dependent opening of large, multi-conductance channels through the mitochondrial outer membrane [61]. In addition, in the hippocampal slice studies discussed above, either OGD or exposure to an Na^+/K^+ ATPase inhibitor (to directly induce SD) caused rapid, Zn^{2+} accumulation in mitochondria, contributing to their depolarization, well before large cytosolic Ca^{2+} rises, onset of SD and cell death [99, 100]. Thus, it seems likely that a critical early effect of Zn^{2+} accumulation under these conditions is to promote metabolic failure, resulting at least in part from mitochondrial interactions. The metabolic failure likely leads to events including SD as well as to Ca^{2+} deregulation, which in turn is directly linked to rapid cellular necrosis.

3.3. Contributions to Physiological Events Associated with Brain Ischemia

A number of physiological processes often come into play in distinct stages after an ischemic episode that can either contribute to the injury process or modify the susceptibility to subsequent ischemia.

3.3.1. Spreading Depression

Among these are the SD events introduced above the occurrence of which seems to contribute to the delayed spread and extension of ischemic brain injury. A recent report found that the occurrence of SD coincided with irreversible loss of synaptic transmission in a hippocampal slice subjected to OGD [104]. In addition to evidence mentioned above that intracellular Zn^{2+} accumulation can contribute to their onset, another recent study found that the occurrence of SD in hippocampal slice was accompanied by a sharp rise in extracellular Zn^{2+} propagating across the tissue. Interestingly, this extracellular Zn^{2+} largely reflected synaptic vesicular release as it was eliminated in slices from ZnT3 knockout mice [105]. This wave of extracellular Zn^{2+} could have complex effects on the evolution of injury, providing a source for

injurious Zn^{2+} entry into cells, but possibly also antagonizing NMDA receptors and decreasing Ca^{2+} dependent effects.

3.3.2. Ischemic Preconditioning

Ischemic preconditioning is a phenomenon whereby a sublethal episode of ischemia triggers changes in the tissue resulting in a decreased susceptibility to injury upon a subsequent ischemic episode. Recent studies have suggested that intracellular Zn^{2+} signals can play a role in the induction of preconditioning. Specifically, in one recent study, Zn^{2+} chelation prevented ischemic preconditioning *in vivo*, presumably after interrupting a sublethal Zn^{2+} dependent injury cascade that would have resulted in decreased sensitivity to the subsequent challenge. In that study, parallel experiments on cultured neurons challenged with Zn^{2+} suggested that the relevant Zn^{2+} dependent effects included P75 activation resulting in caspase-3 activation, HSP70 induction (necessary to halt caspase-3 before apoptosis), and PARP cleavage [106], such that there would be less PARP contributing to injury upon subsequent toxic exposure.

Another intriguing study suggested that a consequence of a Zn^{2+} signal during a precondition stimulus might be an adaptation to limit intracellular Zn^{2+} loading upon a subsequent challenge. Specifically, in this study, “chemical preconditioning” (with cyanide) induced PKC phosphorylation on a specific site of metallothionein, resulting in strong Zn^{2+} rises and activation of a metal regulated transcription factor (MTF-1/MRE), which, via modulation of Zn^{2+} regulatory genes, resulted in decreased Zn^{2+} accumulation and injury upon subsequent excitotoxic exposure [107]. Thus, with sublethal exposure, Zn^{2+} may act in a range of ways to induce protective adaptations.

3.3.3. Reperfusion

A considerable body of evidence supports the idea that reperfusion (restoration of blood flow) after a period of ischemia paradoxically accelerates aspects of the neurodegenerative processes. This is hard to prove unequivocally as the “control condition”, continuous ischemia, is uniformly lethal. However, there is evidence for an oxidative burst upon reperfusion with increasing ROS production [108-110]. One idea is during ischemia, in the absence of O_2 , mitochondria become loaded with excess reduced electron carriers, resulting in an oxidative burst upon sudden restoration of O_2 [109, 111]. Another idea based upon recent studies is that it is the glucose restoration that underlies the reperfusion injury, reflecting glucose driven generation of NADPH via the hexose monophosphate shunt, which provides substrate for NADPH-oxidase, with consequent superoxide formation [112]. Whichever mechanisms predominate, the observation that with “postconditioning”, in which reperfusion is repeatedly interrupted, both the reperfusion associated ROS generation and the resultant injury were decreased, provides intriguing evidence that the reperfusion events can actually enhance neurodegeneration [113]. While direct contributions of Zn^{2+} to reperfusion injury have not yet been examined, it seems likely that Zn^{2+} could contribute, as the oxidative burst would likely trigger an enhanced intracellular Zn^{2+} mobilization, thereby enhancing Zn^{2+} triggered injury processes.

4. Conclusion and Perspectives

The above discussions highlight a host of ways in which Zn²⁺ could interact in various stages of an ischemic injury cascade. We suggest that the most relevant effects of Zn²⁺ vary depending upon factors including tissue characteristics and the duration of ischemia before reperfusion. With brief sublethal ischemia, synaptically released Zn²⁺ may have a net protective effect by attenuating overactivation of NMDA receptors, and mild intracellular Zn²⁺ accumulation may induce preconditioning effects, making the tissue less susceptible to subsequent ischemia. With longer ischemia and toxic accumulation of Zn²⁺ within neurons, mitochondria might be an important early target of Zn²⁺ actions, contributing to metabolic failure underlying Ca²⁺ deregulation and rapid cell death. However, if reperfusion is restored before cell death ensues, other mechanisms may come sequentially into play. First, the reperfusion itself may result in increased Zn²⁺ mobilization and thereby promote downstream Zn²⁺ dependent mechanisms. Zn²⁺ may also contribute to the induction of SD events that can enhance injury. In the post reperfusion period, Zn²⁺ may play important roles in the induction of NADPH oxidase and NOS, resulting in activation of the PARP pathway, which feeds back and interacts with mitochondria as discussed. If neurons are not killed by these pathways, perhaps Zn²⁺ dependent activation of MAP kinases and other signaling pathways comes into play in determining neuronal survival. Or Zn²⁺ may play a role in the late upregulation of Ca-AMPA channels, contributing to late neurodegeneration. Further understanding of Zn²⁺ dynamics and downstream Zn²⁺ dependent events may reveal new targets for effective therapeutic intervention not only in acute ischemia but possibly at stages after reperfusion before degeneration becomes irreversible.

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23. Zinc and the Gastrointestinal Tract

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Abstract. This review focuses on Zn²⁺ transport processes within exocrine cells and mucosal tissues of the gastrointestinal tract. Attention is directed to what is known about mechanisms of Zn²⁺ transport and management within mucosal cells that are functionally unique in each region. An effort has been made to summarize the effects of zinc deficiency on signature functions within each region. Also identified are promising areas for further investigation of zinc transport and management in the gastrointestinal tract, especially with respect to human pathophysiology and disease.

Keywords: zinc, zinc deficiency, ZIP, ZnT, SLC30A, SLC39A, salivary gland, gastrointestinal tract, pancreas, bile.

Introduction

Identified as a “micro”-nutrient, zinc is absorbed in specific regions of the alimentary canal for distribution throughout the body. Secretion of zinc into the lumen is substantial, and its regulation may serve as a response to zinc stores in the body. Recent work indicates that different cell types in different regions express individual programs for uptake, distribution and extrusion or specialized secretion of Zn²⁺. Along with advances in the identification of specific mechanisms of its transport, it appears that Zn²⁺ may play important roles in signature functions within different regions of the gastrointestinal tract. There is also an increasing awareness that a variety of gastrointestinal pathologies may exacerbate or be caused by disturbances of Zn²⁺ homeostasis within cells, tissues and lumen of the gastrointestinal tract. One goal of this chapter is to provide an overview of the role of the gastrointestinal tract in regulating Zn²⁺ handling by the body as a whole. A second goal is to review the known mechanisms of absorption and secretion of Zn²⁺ in the gastrointestinal tract, and explore current research related to the role of Zn²⁺ in the specialized functions of the gastrointestinal tract. A final goal is to review connections between human illnesses and disturbances in Zn²⁺ handling within the cells or lumen of the gastrointestinal tract. This review will focus on exocrine and mucosal functions in the alimentary tract, highlighting areas of particular novelty or evolving investigation.

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1. Systemic Zinc Homeostasis: Regulation by the Gastrointestinal Tract

A syndrome of zinc deficiency was originally recognized by Prasad [1-2] and continues to be a major health problem in indigent populations around the world [3]. Zinc is found in a variety of foods, including red meat, poultry, whole grain cereals, beans, nuts, shellfish, and some dairy products. Intestinal adsorption of dietary Zn²⁺, mechanisms for which are discussed in detail below, is a saturable process. Recent estimates indicate that in young adult males it does not exceed levels 6-7 mg or ~ 100 μmol Zn/day [4], but there is not clear evidence that this ceiling for uptake can be modulated.

The gastrointestinal (GI) tract is also a dominant source of zinc excretion from the body, comprising perhaps 60% of body losses under standardized conditions of normal dietary intake [5]. As summarized in the sections below, zinc is found in high concentrations in saliva, gastric juice, pancreatic secretion and bile. Theoretically, daily excretion could be influenced by the volumes of secretion by each region and by modulation of the composition of each fluid. In addition, in susceptible individuals such as infants suffering from infectious diarrhea or adults responding to massive intestinal resections, losses of zinc could occur independently as a result of increased volume of pathological intestinal secretions [6-8]. Under controlled and non-pathological conditions, it appears that zinc excretion into the alimentary tract is capable of responding to alterations in intake and systemic conditions [5, 9-14].

Measurement of zinc flux into and out of the lumen of the GI tract has been measured by a range of methods. Perhaps the most direct is the measurement of fractional absorption or secretion of stable zinc isotopes. Based on reports of metabolic studies and isotopes loaded into a variety of body compartments, a model of zinc homeostasis has been developed, that permits inferences about fractional uptake and excretion kinetics. The model allows measurement of changes in zinc homeostasis under conditions of zinc excess or deprivation, along with various disease states, without the risks inherent to the use of radioactive isotopes, allowing for safe studies in human subjects [4, 15]. In humans, such studies indicate that, acutely, whole body zinc homeostasis can be maintained under conditions of both increased and decreased zinc ingestion over a wide range of dietary zinc intake (22 to 306 μmol Zn/day) through modulation of uptake or excretion over a period of 4-12 days [5, 14]. Studies in humans and rats confirm that intestinal zinc absorption can approach 100% efficiency under conditions of low zinc intake [5]. Cousins and others have reported and summarized [11] dietary constituents that might influence absorption of Zn²⁺ in the gastrointestinal tract (See Chapter 3 for details).

Rates of fecal excretion, primarily from biliopancreatic secretions, can range from 0.1 μmol/day to 3 μmol/day, offering the possibility that systemic homeostasis is thereby being regulated [16]. At the same time, the exchangeable pool of zinc in the body is limited [14] and is not easily maintained during prolonged periods of dietary deprivation of zinc [17]. Zinc handling within the gastrointestinal tract is influenced by stage of life. The newborn has special requirements for zinc, which is required to support rapid body growth as well as immune and cognitive development. Human milk contains high concentrations of zinc immediately post-partum (0.5 mg/L to 3 mg/L) to support this demand, which comprises almost 25% of daily maternal intake [18-20]. Scattered reports indicate that other special components of breast milk, such as lactose, citrate, or casein may facilitate or hinder absorption in different regions of the alimentary tract in the neonate [21-23]. In infants, a syndrome of deficiency may

develop rapidly, due either to absence of adequate intake of zinc by the nursing mother or because of mutations in zinc transport proteins in lactating mammary gland [19, 24]. In the elderly, intestinal capacity for Zn^{2+} absorption appears to be decreased compared with young adults [25-26], a finding confirmed in experimental animals [27]. At both extremes of age, it may be predicted that prolonged deficiencies in dietary zinc will be exacerbated by obligate losses from the gastrointestinal tract.

2. Mechanisms of Absorption and Secretion of Zn^{2+} in the Gastrointestinal Tract

2.1 Zinc Absorption in the GI Tract

2.1.1. Distribution of Zn^{2+} : Uptake and Uptake Kinetics.

Zinc isotope uptake studies have demonstrated that zinc absorption occurs primarily in the duodenum (60%), ileum (30%) and jejunum (10%), with minimal absorption in the large intestine [28]. Uptake is regulated both by molecular mechanisms on the apical and basolateral surfaces of the enterocytes, and by association with other dietary factors that either promote (e.g. breast milk, animal protein) or retard (e.g. phytate) zinc absorption [13]. Kinetics of zinc absorption are governed largely by both saturable, carrier-mediated processes, but non-saturable processes may be present under some circumstances [29].

2.1.2. Confounding Factors in Assessment of Zn^{2+} Absorption by the Gastrointestinal Tract.

A number of factors influence apparent rates of intestinal uptake of dietary Zn^{2+} , thereby obscuring evaluation under pathologic circumstances. One is appetite, which is linked, at least indirectly, with dietary intake. Thus it is useful to recognize that dietary zinc deficiency is associated with anorexia, in many species. Reeves and colleagues have described a specific decrease in protein intake in rats in response to short-term zinc deprivation, with rats choosing lower protein diets and consuming fewer calories overall. [30-31]. In addition, impairments of taste and smell are associated with systemic deficiency in Zn^{2+} and can respond to supplementation in the diet [32-33].

A second confounding factor is the presence of dietary components that influence availability of Zn^{2+} . Animal products provide more predictable levels of bio-available zinc than do those of plants. In plant sources, Zn^{2+} is often associated with phytates that have high binding capacity for divalent cations such as Zn^{2+} and that can considerably modulate the fraction of dietary Zn that is absorbed [4]. In a phytate-free meal, current recommendations for meeting physiologic requirements in men, which are in the range of 3 to 4 mg Zn /day, might be met by a dietary intake of 8 to 10 mg Zn/day. In computerized models, with a daily intake of 900 mg phytate, daily intake of zinc must be in the range of 100 mg to achieve close to 6mg/day adsorption, emphasizing the profound impact of non-animal food sources on the pool of dietary zinc that is accessible for absorption [4].

A third confounding factor is the presence of other metals that can compete with or influence avidity of absorption processes for Zn^{2+} . These would include Cu^{2+} , $Fe^{2+}/3+$ Cd^{2+} , Mn^{2+} or Ni^{2+} . Zinc has long been known to compete with copper in intestinal absorption, with zinc supplementation leading to copper deficiency and vice versa.

Increased dietary zinc increases the expression of metallothionein which has a higher affinity for copper than it does zinc which constrains copper within the cells of the mucosa. [34] These effects appear to be limited to conditions of very high zinc or copper concentrations above what would be expected in the common diet [35]. Similarly, zinc depletion (along with depletion of calcium and iron) allows increased absorption and accumulation of cadmium in the GI tract, while adequate zinc intake increases the turnover of cadmium [36].

A final confounding factor relates to unsuspected variations or alterations in luminal conditions, even in apparently normal subjects who might be taking prescription or over-the-counter medications. For example, impairment of Zn²⁺ absorption is a documented concern of long-term use of medications (proton pump inhibitors, PPIs) that inhibit gastric acid secretion [37]. The clinical significance of this impairment is unclear for otherwise healthy adults [37-38]. However, it may turn out that chronic use of PPIs would more significantly alter handling of zinc within the gastrointestinal tract in populations at higher risk for dietary zinc deficiency. This may be particularly true for the elderly, for whom zinc intake is low and PPIs are quite freely prescribed or available over-the-counter for unsupervised use [39].

2.1.3. Associations with Transport Proteins: Identification and Mechanisms.

Over the years, transporters and channels have been identified in different regions of the intestine, with evidence supporting some capacity for transport of Zn²⁺ and suggestions that they may be involved in vectorial transport across the intestinal epithelium. In 1997, a polyfunctional metal transporter, currently known as DMT-1 was identified in the duodenum [40]. It plays a major role in intestinal transport of Fe²⁺ [41]. Initial studies indicated the presence of Zn²⁺-induced currents, which now seem attributable to movements of H⁺ and Cl⁻ but not Zn²⁺ [42]. Recent studies [43] have also suggested permeation of Zn²⁺ though a channel, TRPV6, a member of the vanilloid receptor family originally cloned from rat duodenum. In this study [43], influx of Zn²⁺ was inferred by monitoring electrical currents and increasing fluorescence of a non-specific reporter, fura-2, which is sensitive to Zn²⁺ when present in different epithelial cells of the gastrointestinal tract [44-45]. The design of the study, however, leaves open the question whether the observed effects in fura-2 were due to an induced intracellular release of intracellular Ca²⁺, rather than actual permeation by Zn²⁺. A common challenge for studies evaluating the promiscuity of such transporters for multiple ions is to utilize specific reporters and chelators, with binding affinities appropriate to the conditions that are physiologically or pathologically relevant. An additional challenge is to utilize realistic conditions for monitoring permeation of Zn²⁺, which may well be present in its labile, or loosely bound form, in micromolar concentrations in some luminal environments, but which may be present only at very low levels when high affinity binders are present.

With respect to specific zinc transporters and as explored throughout this book, two families have been identified, the SLC30A or ZnT family, which exports zinc from the cytoplasm, and then SLC39A or Zip family, which imports zinc into the cytoplasm. Each family has multiple members, some of which have been characterized, and others whose existence has only been predicted based on sequence analysis. With regard to transporters involved in intestinal absorption, dietary content and systemic zinc status have been shown to regulate expression of these transporters. In addition, most transporters are localized within the cell, serving to distribute Zn²⁺ to or from

intracellular compartments. These aspects of intracellular localization and potential contributions to intracellular distribution have been reviewed recently in great detail [9, 46] (see also Chapter 8).

With regard to pathways for vectorial transport (i.e., lumen to nutrient) of Zn^{2+} , a great deal remains to be learned. In mature rats, expression of ZnT-1, the prototype zinc export protein, is detected on the basal/lateral surfaces of enterocytes and is amplified in response to non-acute dietary supplementation with bioavailable zinc [47]. In contrast, the zinc import protein for ZIP-4 localizes to the apical surface of absorptive enterocytes in the villus tips, but not in the crypts in mouse small intestine under conditions of zinc depletion and is up-regulated in response to systemic zinc deficiency [9, 48-51]. This up-regulation is rapidly reversed when dietary zinc is repleted. Also demonstrated is decreased expression of zinc exporters ZnT-2 and ZnT-4 which serve to sequester zinc into specific intracellular compartments within enterocytes under the same conditions. [50].

In the colon, conflicting results have been reported with respect to the ability of the colon to absorb Zn^{2+} , a circumstance that may be explained by differences in species, developmental stage and age, region studied, or bacterial ecology in the lumen [52-55]. Accordingly, the functional characterization of ZnT and ZIP profiles is only partially developed for the colon. Among the SLC39A (ZIP) family, mRNA for SLC39A5 (ZIP-5) was detected in colon tissue, not separated by mucosa or muscular layers [56]. In epithelial cells of the small intestinal crypts and model epithelial cell culture systems, ZIP-5 localizes to the basolateral membrane [56-57], implying that this would also be true for epithelial cells of the colon. In addition, expression of ZIP-5 is unaltered during conditions of zinc deficiency. In contrast, ZIP-4 is expressed apically in the colon's absorptive surface cells and not so clearly in crypts, and is up-regulated in response to dietary zinc deprivation [57]. With regard to the ZnT exporter family, Yu et al. [58] reported apical localization of ZnT-6 and lateral membrane localization of ZnT-1 in the mouse cecum and large intestine, suggesting potential avenues of extrusion. Models of Zn^{2+} absorption or secretion may thus be proposed for the colon surface epithelium, as for the cells of the crypts and villi of the small intestine.

In the intestine and colon, expression profiles of ZnTs and ZIPs seem to be connected to tissue function, reflecting absorptive activities or mucosal functions that influence luminal conditions. Based on a variety of contributions in the literature [46, 50, 56-61], Wang and Zhou recently updated a model for management (uptake, distribution, excretion) of Zn^{2+} in the model absorptive enterocyte (Figure 1), in which ZIP-4 dominates as the mechanism of import/absorption across the apical membrane, ZIP-5 dominates as the mechanism of uptake across the basolateral membrane, and ZnT-1 dominates as the mechanism of outflow across the basolateral membrane.

One important caveat is that this formulation is the need to more fully understand the contributions of other transporters, the conditions that govern transcription and translation of different transporters, and potential variations in expression at different stages of life. In this regard, Jou et al [62] recently published a meticulous exploration of expression of different transporters in response to different levels of dietary zinc depletion, in weanling rats. In their studies, the ZIP-4 protein was found throughout the length of the villus, in contrast to localization in the apical portions in adult animals. In addition, ZnT-1 protein localized to both apical and lateral surfaces of enterocytes, and ZnT-5 was detected in apical membranes in crypts and tips of the villi. In this study, the authors also demonstrate discordance of mRNA levels and protein expression, in response to varying levels of dietary zinc deficiency. They observe that intestinal

expression of protein for ZIP-4 is not nearly as responsive to zinc deficiency as are its mRNA levels, whereas expression of protein for ZnT-1 is much more sensitive than are its mRNA levels [62]. With respect to coupled- or counter-ions of transport, it is interesting to note that, in its original description by Palmiter and Findley[63], ZnT-1 mediated efflux of $^{65}\text{Zn}^{2+}$ was not influenced by alterations in extracellular Na^+ , K^+ , Mg^{2+} , Ca^{2+} or Cl^- , arguing against transport coupled to these ions or to changes in membrane potential that would have been elicited by increases in extracellular K^+ . We have not been able to identify published studies exploring whether ZnT-1 mediated extrusion of Zn^{2+} might be coupled to inward movements of H^+ , a mechanism which has been proposed in other cell types for other members of the SLC30A (ZnT) family [64-66].

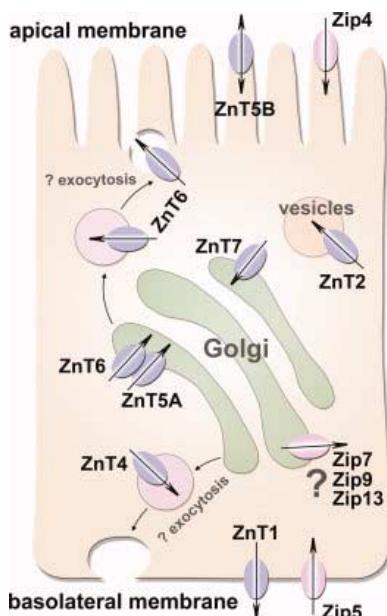


Figure 1: Zinc transport and secretion in a model enterocyte (From Wang and Zhou [46], with permission).

Similarly, there is a need for a systematic exploration of the ionic conditions and coupling ions for transport in the SLC39A (ZIP) family in intestinal cells. In isolated brush border preparations from pig intestine, Tacnet, et al. [67] noted that absorption was sensitive to pH within the absorptive vesicle, but not under perturbations consistent with counter-transport with H^+ or co-transport with Cl^- or HCO_3^- . In fact, their studies were most consistent with transmembrane carriage in connection with small peptides such as Gly-Gly-His [67]. In addition, our recent studies [68] provide evidence that basolateral import of Zn^{2+} into cells of the gastric epithelium may occur in exchange for cytoplasm Ca^{2+} ions, a possibility that may apply to some members of the ZIP family. However, formal connections of coupling ions have not been rigorously established.

2.1.4. Connections between Zinc and Signature Functions in Absorptive Regions of the Gastrointestinal Tract.

Zinc in the Small Intestine. Zinc plays an essential role in intestinal mucosal development during infancy. Weanling rats fed zinc deficient diets maintain intestinal weight and length but exhibit reduced expression of digestive enzymes including sucrase, maltase, lactase, leucine aminopeptidase, and alkaline phosphatase [69]. Similar results have been described in weanling pigs, where addition of organic zinc to the diets of feeding sows lead to increased villus height and width throughout the small intestine of their offspring[70] Zinc supplementation also leads to increased crypt cell turnover and accelerated crypt cell development in mice[71]. Similar effects of zinc have been observed in adult animals. Dietary deficiencies in zinc also lead to reduced efficiency of intestinal absorption of water, electrolytes and glucose [72]. Zinc-depleted rats have marked decreases in the activity of disaccharidases and alkaline phosphatase, suggesting that zinc deficiency may impair digestion of carbohydrates[73]. Impaired carbohydrate metabolism may contribute to the nutritional deficiencies and diarrhea associated with zinc depletion [74]. Rats fed zinc-deficient diets exhibit a similar impairment in the uptake of carbohydrates [75]. Such observations testify to the importance of normal body stores of Zn^{2+} in maintaining a proper digestive milieu in the small intestine, but do not directly address a role for Zn^{2+} in regulating absorption of nutrients or influencing the luminal microenvironment.

Zinc in the Colon. As in the small intestine, there are lines of evidence suggesting that systemic deficiencies in Zn^{2+} can lead to disturbances in transport [53] and neurohumoral regulation [76] and increased availability may lead to proliferation of mucin-producing goblet cells [77]. Relatively little information is available related to the role of endogenous zinc in regulating normal colon mucosal transport. Apical chloride conductance, thought to be crucial in mediating colon secretion in response to toxicogenic agents appears to be inhibited by luminal Zn^{2+} at $\sim 100\mu M$ [78-79]. Such concentrations may be in the range for the lumen or microenvironment near the surface epithelium, and might thus be physiologically relevant.

With respect to “signature” functions of intestinal epithelial cells two lines of evidence are beginning to emerge related to roles for Zn^{2+} in regulating mucosal function or luminal conditions. The first relates to the description of an extracellular receptor for Zn^{2+} , ZnR, for which evidence was first organized by Hirshfinkel and colleagues in the HT-29 colon cell line [80-81]. In their studies, exposure to extracellular concentrations of Zn^{2+} in the sub-millimolar range led to release of intracellular Ca^{2+} from intracellular IP₃ (inositol 1,4,5 triphosphate)-sensitive stores; to activation of pathways connecting MAP Kinase, PI3 Kinase and ERK1/2; and to enhanced membrane $Na^+ - H^+$ exchange. In follow-up studies, application of Zn^{2+} mitigates intracellular acidification caused by exposure to the short chain fatty acid, butyrate, a major product of bacterial metabolism in the colon [82]. Protective and paracrine effects in different cell lines have also been reported, providing a conceptual framework for postulating that ZnR may integrate a number of mucosal functions and barrier properties [82]. This line of investigation is highly provocative. It awaits isolation, cloning, and definitive localization of ZnR, as well as exploration of the role of Zn^{2+} a first messenger, as has been done for the extracellular Ca^{2+} receptor [83-85].

A second most interesting line of investigation has opened up with respect to the Paneth cells in the intestinal crypts. Paneth cells are important contributors to the intestinal antimicrobial barrier through synthesis and release of antimicrobial peptides

and proteins [86-87]. Animal studies indicate that Paneth cell numbers, location and granule morphology are altered by infection and mediator of systemic stress such as corticosteroids [86, 88]. Connections between zinc dysregulation and Paneth cells were first reported in patients with acrodermatitis enteropathica [48, 89], a disease now attributed to genetic mutations in ZIP-4 and defective absorption of zinc [49, 90]. Subsequent studies have shown that the secretory granules of Paneth cells contain high content of zinc and are especially sensitive to acute deprivation of Zn²⁺ in the diet [91]. Paneth cells degenerate rapidly and selectively after systemic administration of zinc chelators such as dithizone [92]. Using dynamic imaging methods with fluorescent reporters, Giblin et al. have shown secretion of Zn²⁺ into the lumen of the intestinal crypt that is selectively responsive to some but not all muscarinic agonists [93]. It is not yet known whether and how zinc content of the secretory granule may be connected to its anti-microbial and pro-inflammatory functions.

2.2 Zinc Secretion in the GI Tract.

As outlined above, Zn²⁺ is made available to the body through ingestion of foods that may vary quite widely in content and biological accessibility. Over time, ingestion of these foods, along with other conditions, may influence the receptiveness and capacity of the small and large intestine to absorb Zn²⁺, as a means of regulating Zn²⁺ homeostasis within the organism. In this context, there may be significant contributions to excretion rates for Zn²⁺ from active secretions by specific organs, which do not themselves have a clear capacity for absorption. These organs include the salivary glands, the stomach, the pancreas and the biliary tract. Table 1 provides summaries of measurements of the Zn content in such secretions. Each organ releases significant, but quantitatively different, levels of Zn²⁺ with its secretion, or juice. Because individual organs function so differently, each will be discussed separately with respect to mechanisms of secretion and physiological impact of secreted Zn²⁺.

2.2.1. Salivary Gland Secretion

Saliva is essential for preparation of a bolus of food for transmission to stomach through the stomach. The preparation of the bolus requires warming, hydration, and lubrication. Digestion of lipids and carbohydrates is initiated, as is the release of certain nitrite and nitrate compounds that ultimately contribute to the bactericidal environment within the lumen of the upper GI tract [103]. Apart from enzymes of digestion, several classes of bioactive substances are found in human saliva, including mucins, defensive enzymes, growth factors, endocrine and paracrine signaling molecules [104-105]. In addition, the saliva optimizes receptiveness of the tongue and oral cavity for the sensations of taste and smell [106].

There is some evidence in both humans [107] [108] and experimental animals [108] that systemic deficiency in zinc is associated with impaired secretion rates for saliva and may contribute to alterations in bacterial ecology of the oral cavity [109-110]. In a model of severe and sustained dietary zinc deficiency (0.9ppm for 4 weeks) in the rat, Johnson and Alvarez [111] noted decreased production of proline-rich salivary proteins but increased production of digestive enzymes such as amylase and deoxyribonuclease, even controlling for impairments in growth and weight gain. In other studies, deficiencies of dietary zinc have been associated with decreased production of mature secretory granules and defective responsiveness to secretory agonists such as

acetylcholine[112]. Intracellular proteins and enzymes participating in energy production are also deficient [113].

Table 1. Content of Zinc in Secretions from Different Regions of the GI Tract

REGION	Age Range Gender (M:F) Condition	Zn content	Method of Measurement	Ref.	Comments
Saliva	278 adults 120:158		Flame AA Parotid secretion from cannulated duct	[94]	Perhaps age and sex-related differences are present
	Mixed Sediment	0.96(0.34-3.10) µmol/g			
	Mixed Supernat	0.61 (0.15-1.67) nmol/L			
	Parotid duct	0.89 (0.24-4.86) nmol/L			
	6 adults Mixed saliva	0.11±0.11 nmol/L	Zinquin (measures labile pool)	[95]	No direct comparison of labile to total content
Stomach	44 adults 15:29	55± 17 µg/L	Electrothermal atomic absorption spectrometry	[96]	Levels in Males >Female
	28 adults 17:11		ICP-ES (ICP-MS)	[97- 98]	Precautions against contamination by saliva and duodenal content taken
	Control	13.0± 0.6 µmol/L			
Pancreas	Peptic ulcer (6DU, 5GU)	12.1±2.5 µmol/L			
	20 to 68 yrs 11:8 baseline	5.0 ± 0.7 µmol/L	Flameless AA Endoscopic cannulation	[98]	Not diseased (5) Early cancer (9) Pancreatitis (5)
Bile	N=25 Control	1.8 ± 0.6	ICAP (ICP-MS) (aspirates)	[99]	Zn content inversely correlates to content of lipid and protein
	Pigment stone	1.8 ± 0.3			
	Cholest. stone	1.2 ± 0.3 (µmol/ml)			
	N=3 2:1	1- 4 µg/ml	Flameless AA (Surgical drains)	[100]	Content declines following operation
	N=43 Bile in GB Bile from liver	0.3 ± 0.5µg/ml 1.1 ± 0.8 µg/ml	Flameless AA Surgical samples	[101]	Zn content in GB bile lower than in hepatic bile
	N=48 patients 20 control (surgery not for stones or cancer)	0.94± 0.12 µg/ml	Flameless AA	[102]	Zn content in pigment and cholesterol stones not significantly different (range 20 to 60 ppm)
	N=28 patients with gallstones	1.56 ± 0.16 µg/ml			

It seems that the presence of independently secreted Zn²⁺ may be important for some functions of saliva, including taste for salt and bitter sensations [108, 114-118]. These functions appear to be transduced at least partly through the actions of gustin, also known as carbonic anhydrase VI [118]. Somewhat surprisingly, viscosity and mucin structures within saliva may not be influenced by content of Zn²⁺ as compared to other divalent cations. [119]. One area of evolving interest is the connection of Zn²⁺ to antimicrobial functions of saliva [120-123]. In particular, histatin-5, a histidine-rich protein in salivary secretion, appears to have important cidal properties against both candida [120] and gram-positive bacteria [123]. Histatin-5 binds Zn²⁺ with loose affinity (1.2×10^{-5} M) suggesting of the possibility that variations in levels of salivary Zn²⁺ might regulate activity [120]; moreover, synthetic analogues of this protein retain anti-microbial activity that requires Zn²⁺ [122-123].

Very little information is available regarding the mechanisms of vectorial (i.e., basolateral to apical) transport of Zn²⁺ in acini or ducts of the salivary gland system or the role of Zn²⁺ in regulation of salivary secretion. In rat acini and ducts, evidence has been presented to suggest that levels of extracellular Zn²⁺, in the low micromolar range, may inhibit, without permeating, store-operated channels for extracellular Ca²⁺ [124]. Such observations offer the possibility that extracellular Zn²⁺ might modulate secretory functions of salivary acini or ducts through alterations in Ca²⁺ store-refilling. It is not clear, however, under what conditions concentrations of free or loosely bound Zn²⁺ on the nutrient side of the epithelium approach or exceed those detected in the salivary secretion itself.

2.2.2. Gastric Secretion.

The gastric mucosa is divided into two spatially and functionally distinct but connected regions: the gastric glands and the surface epithelium. The signature secretion product of the stomach is hydrochloric acid, secreted into the lumen by parietal cells in the complex gastric gland [125-126]. Pepsinogen and other digestive enzymes are secreted from Chief cells at the bases of the glands, and its conversion to active Pepsin A is auto-catalyzed in the acidic luminal environment. Subpopulations of parietal cells also secrete various growth factors [126] and intrinsic factor, which binds dietary vitamin B12 is also secreted located in the midportion of the gastric gland [127]. Additional secretion products of the gastric gland include antibacterial c-type lysozyme [121, 128] which harbor a direct muramidase enzymatic function and a direct cidal function. Lysozyme, a highly basic molecule ($pK_a > 9$), is effective against gram positive organisms but less effective against gram negative organisms [121]. Bacterocidal fragments of lysozyme, are generated by the actions of pepsin [128]; however, although the structures of such fragments are stable, their activities are inhibited at acidic pH [129] and only become active when gastric contents are alkalized. These considerations suggest secretion of acid and pepsin permit the generation of bactericidal lysozymes which would then become active in the resting stomach--when luminal pH increases but oral flora are routinely swallowed.

Non-acidic secretions are also detected from the gastric surface epithelium and the adjacent and closely connected duodenal surface epithelium. Most prominent among these secretions are mucus glycoproteins, surfactants and phospholipids, and bicarbonate (Na⁺ - HCO₃⁻) [130-131]. A prevailing conceptual framework is that the surface epithelium protects the underlying tissues from the damaging effects of luminal acid and other noxious stimuli [125, 130].

Deficiencies in Zn²⁺ can influence mucosal function and integrity in the stomach. In experimental animals, Zn²⁺ deficiency amplifies injury [132], retards healing of ulcers [133] and promotes esophagogastric carcinogenesis [134]. Curiously, in animal models of graded dietary zinc deficiency, secretion of acid and pepsinogen were reportedly increased [132]. Conversely, it has been recognized for many years that preparations containing zinc accelerated healing of injury and ulcers in the upper gastrointestinal tract [135]. Acid secretion appears to be suppressed during administration of such zinc preparations [136-137], along with improvements in production of tissue levels of metallothioneins, mucus and endogenous, protective prostaglandin E2 [138-139]. Without providing a clear picture of the mechanisms involved, these findings implicate Zn²⁺ in ongoing processes of secretion, protection and mucosal renewal.

In recent years, direct evidence for regulation of Zn²⁺ content in gastric secretions has begun to emerge. The stomach is not normally a significant site of absorption of Zn²⁺ [52, 140]. At the same time, very significant concentrations of Zn²⁺, in the range of 10 micromol/L, are detected in gastric juice not contaminated by oral intake, saliva or duodenal reflux [97]. With regard to the mechanism of its appearance in gastric secretions, much is not yet clear. Based on the absence of a relationship between luminal pH and Zn²⁺ content, Powell et al. [97] suggested that movements of Zn²⁺ into the lumen are passive and dependent on concentration in the serum. One argument against this hypothesis is that the stomach is an electrically and morphologically "tight" epithelium [141], meaning that its intercellular junctions are permeable only to small and nonpolar solutes [142] or to very high concentrations of monovalent ions [143] that disrupt mucosal integrity. The epithelium is not normally permeable to divalent cations, including Zn²⁺ [144] at millimol/L concentrations. A second argument is that "free" or even loosely bound concentrations of Zn²⁺ in plasma have been both predicted and measured to be much lower than the labile concentrations detected in gastric juice [145], due to very high affinity binding proteins such as albumin and alpha-2 macroglobulin.

It seems likely that transport of Zn²⁺ into the lumen occurs through the cellular pathway [65, 146]. After appearing in the lumen, Zn²⁺ probably associates with binding molecules of varying affinity, from small organic acids to large mucins [147]. Although luminal pH does not correlate directly with content of Zn²⁺ in the gastric juice [97], it is likely to play a significant role in regulating the binding affinity of these molecules for Zn²⁺ [147]. The relationship has been demonstrated most clearly with oral zinc preparations used to treat gastritis and peptic ulcer conditions [148], where affinity of Zn²⁺ and its binder (in this case L-carnosine) is increased at neutral pH and greatly diminished at acidic pH.

Our studies in isolated rabbit gastric glands have suggested that content of Zn²⁺ within the secretory compartment of the parietal cell is required in order to maintain its highly acidic microenvironment [146]. Subsequent studies provided evidence that secretory stimulation leads to greatly enhanced uptake of Zn²⁺ across the basolateral membrane of the parietal cell [64], suggesting that apical secretion is coupled to a mechanism that regulates demand for Zn²⁺ across the basolateral membrane. Expression of the Zn²⁺ exporters, ZnT-4 and ZnT-5, has been shown in mouse parietal cells using immunohistochemical staining methods [58]. In highly purified preparations of apical membrane and secretory tubulovesicles obtained from gastric glands in the rabbit, we have shown enrichment of ZnT-5, providing additional evidence that it is a likely mechanism of transport across the apical membrane into the gastric lumen [65]. In

addition, ZnT-1 was not detected in any cells of the gastric gland, whereas ZnT-6 was detected in chief cells and ZnT-7 in mucus neck cells. Detailed exploration of ZIP proteins has not yet been reported for the stomach as a whole or in specific cell populations of the gastric glands, nor has there been a report of systematic exploration of ZnT or ZIP transporter families in the surface epithelium.

2.2.3. Exocrine Pancreatic Secretion.

The exocrine pancreas is organized spatially and functionally as are the salivary glands. The gland is divided into lobules, containing smaller and smaller branching units until the terminal functional unit, the acinus. Ductal structures from these units converge ultimately to empty into the main pancreatic duct. Acinar secretion occurs in response to a variety of neuroendocrine stimuli and involves emptying of zymogen granules that include a plethora of digestive enzymes [149]. Major ductal secretions include mucus and HCO_3^- [150]. Content of Zn^{2+} in pancreatic juice, reported in the range of ~5 micromol/L [98], appears to reflect the protein-rich acinar content almost exclusively [151-152], although contributions from ductal secretion and/or absorption have not been fully excluded. Within the acinar cells, high content of Zn^{2+} has been visualized in zymogen granules, using autometallography [153]. The nearby pancreatic islets, which also contain concentrations of Zn^{2+} in secretory compartments.

Identity of the transporters involved in secretion of Zn^{2+} by the acinar cell into the lumen of the acinus has been reported. Recent studies implicate ZIP-4 and ZIP-5 as transporters localizing to the basolateral membrane and responsive, under different conditions, to the cell's demand for Zn^{2+} from the nutrient compartment [50, 56-57, 59]. Guo and Cousins [59] have proposed a model for zinc management in the pancreatic acinar cell, which focuses on ZIP-4 as the dominant mechanism responding to the demand for Zn^{2+} from the nutrient side and on ZnT-1 and ZnT-2 as the likely mechanisms of apical transport. They propose that ZnT-1 mediates movements directly across the apical membrane, while ZnT-2 provides a channel of uptake for Zn^{2+} into the secretory granules (Figure 2). They also provide evidence for regulation of Zn^{2+} transport to the granules (ZnT-2) through signaling pathways that include metal responsive elements and through members of the family of signal transducers and activator of transcription (STAT), specifically, STAT-5 [59]. From this model emerges a picture of zinc management, run much like train depot where multiple trains with specific (perhaps pre-assigned) destinations enter and leave, occasionally requiring acceleration, switches or stops to maintain proper flow and prevent accidents.

Moderate depletion of dietary Zn^{2+} (<10 ppm for 4 weeks, not associated with signs of systemic deficiency) leads to severe depletion of Zn^{2+} in exocrine zymogen compartments, as compared to the endocrine secretory compartments of the beta cells [151-152]. More severe or prolonged depletion of Zn^{2+} leads to severe morphological disturbances in zymogen granules and acini, in addition to causing impairment of volume and protein content in pancreatic ductal secretion [154-155]. Curiously, severe depletion may lead to up-regulation and secretion of subgroups of enzymes: serine proteases such as trypsinogen and chymotrypsinogen [155], enzymes that have been implicated in early cellular events of acute pancreatitis and in idiopathic chronic pancreatitis [156] [157-158]. Conversely, moderately elevated content of zinc in the diet may preferentially lead to uptake, morphologic and functional disturbances [159-160]. While not yet clearly connected to human disease or pathophysiology, these

observations indicate the high level of responsiveness and sensitivity of the exocrine pancreas to alterations in systemic zinc homeostasis and potentially systemic stress [59]

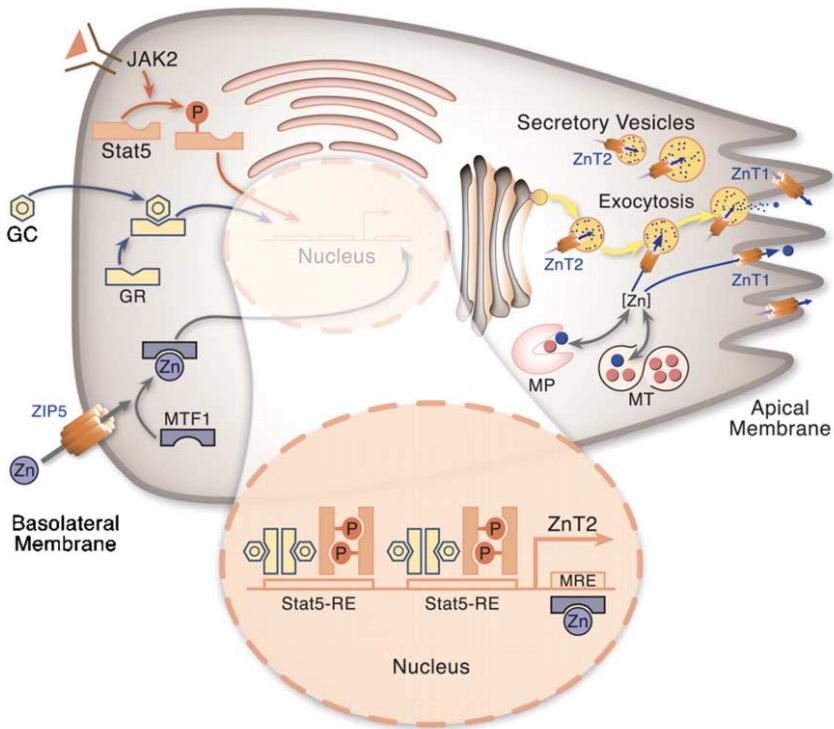


Figure 2: Proposed model of zinc transport in pancreatic acinar cell secretion From [59], with permission)

The role of Zn^{2+} within the pancreatic juice remains a target of speculation. For many years it has been recognized that Zn^{2+} is structurally integral to specific enzymes that may be harvested from pancreatic juice [161], fitting the definition of metalloproteins [162]. Along the pathway of zymogen synthesis and secretory granule maturation, the precise point of Zn^{2+} incorporation remains unclear. Based on work in other secretory cells, it could be as early as the phase of protein translation and assembly in the endoplasmic reticulum or Golgi [163-164]. Additionally speculative is whether Zn^{2+} might play a role in maintaining the integrity or internal microenvironment of the secretory granule, where a link between acidity and Zn^{2+} content [64-65, 146] remains to be explored.

Over the years it has been recognized that Zn^{2+} ions may impair activity of digestive enzymes in separate interactions [161, 165-166]. An intensively studied case of such metal-protein interaction is that of carboxypeptidase A [165-166], a Zn^{2+} -containing metalloenzyme that also has a distinct inhibitory interaction with Zn^{2+} . This interaction occurs in a range ($K_i \sim 0.7 \mu M$) that would be physiologically relevant within the zymogen granule or exocrine juice; it is also pH dependent, such that inhibition by Zn^{2+} seems to optimize at neutral pH. Under physiologic conditions zymogen granules are acidic with respect to the cytoplasm [167], perhaps making this form of inhibition irrelevant. However, these considerations offer the possibility that when initially

released into the lumen of the pancreatic duct, high concentrations of Zn²⁺ in the micromolar range would competitively inhibit enzyme activity and efficiency [165] until extracted by metallothionein that is also secreted into the lumen [168] or diluted in the general mixing that would occur in the duodenum following a meal.

2.2.4. Biliary Secretion.

The liver is a major repository for Zn²⁺ in the body and it is not surprising that Zn²⁺ is excreted in bile, perhaps only because it cannot be recycled or perhaps in response to changes in systemic homeostasis [99-101]. Mechanisms of Zn²⁺ transport in hepatocytes and the reticulendothelial system are beyond the scope of this chapter but are discussed in detail in Chapter 24.. Levels of Zn²⁺ in bile are likely to be sensitive to systemic zinc homeostasis, including changes caused by diet, acute stress or malignancy [169-170]. Curiously, under baseline conditions, it appears that levels of Zn²⁺ are lower in gallbladder bile than in hepatic bile [101], offering the possibility of absorption by the epithelium or adsorption by mucins tightly adherent to the epithelium. In this regard, a divalent metal transporter (DMT-1) [40] has been detected on the luminal surface of biliary tract epithelial cells [171], offering a possible mechanism for Zn²⁺ absorption before bile reaches its storage site in the gallbladder. It remains unclear, however, whether Zn²⁺ is a physiologically relevant substrate for this transporter [41-42]. To our knowledge, there have been no reports providing details of ZIP (SLC39A) or ZnT (SLC30A) expression in cholangiocytes of the bile duct or gallbladder. In addition, in one early report, it was suggested that high concentrations of Zn²⁺ (2.5mM) in the lumen may interfere with apical membrane anion exchange, suggesting a possible influence of fluid absorption [172]. Otherwise, a physiologic role for Zn²⁺ in bile is very much open to exploration.

3 Zinc in Disease of the Gastrointestinal Tract

3.1 Systemic Zinc Dys-homeostasis and GI Function

Zinc deficiency can be overt in undernourished patients or present as a hidden form of malnutrition in susceptible populations [3]. As outlined in prior sections, deficiencies in body zinc pools can lead to impairment of secretory processes and defects in products of secretion. As noted above, systemic zinc deficiencies lead to impaired smell, taste and appetite, as well as diminished volume and content of salivary secretion. Curiously, gastric acid secretion is amplified during zinc deficiency [132], and inhibited by direct ingestion of zinc preparations [136-137], raising the possibility that acid secretion might enhance liberation from binders of Zn²⁺ in chyme or efficiency of Zn²⁺ absorption in downstream regions. Severe zinc deficiencies have been associated with diarrhea, which itself can be associated with excess wasting of Zn²⁺ with high outputs of stool volume [6-8]. The cause of diarrhea in the setting of zinc deficiency is likely multifactorial. As noted above, zinc deficiency decrease secretion of digestive enzymes, impair processes of electrolyte and water absorption, and exacerbate deficiencies in protein synthesis [6, 69, 72, 74-75, 77, 173-174]. A number of properties of both mucosa and luminal microenvironment may also be affected by zinc deficiencies such as alterations in luminal microflora and oxidative stress [3, 73, 132, 174-175]. It seems

likely that systemic impairments of innate immunity also could play a role in lower susceptibility to intestinal pathogens.

Diarrhea may benefit from zinc supplementation, though prolonged supplementation of zinc can lead to unintended consequences, such as copper depletion [176]. A number of reviews and analyses have recently been published, suggesting that children in under-developed regions may benefit from oral zinc supplementation, whether or not there is evidence of systemic zinc deficiency (see Chapter 11) [177-179]. In adults with various forms of diarrhea the therapeutic benefit of such supplementation is not likely, unless there is clear evidence of zinc deficiency. In children or adults with HIV and diarrhea associated with zinc deficiency, significant improvements in diarrhea are observed [180].

3.2 Dysregulation of Zinc Transport in the Gastrointestinal Tract

3.2.1. *Acrodermatitis enteropathica*

In the past few years, there has been one well-documented example of a genetic disorder of zinc transport in the gastrointestinal tract that leads to a recognized human disease. As noted above, mutations in ZIP4 (SLC39A4 gene located at 8q24.3) are causally associated with acrodermatitis enteropathica, a syndrome of zinc deficiency characterized by growth retardation, immune system dysfunction, alopecia and dermatitis, diarrhea and cognitive dysfunction [49, 181]. Usual presentation is in infancy, but can be as late as adolescence. If recognized early, many of the manifestations of disease can be arrested or even reversed with oral supplementation, but responses are not uniformly observed [48-49, 181]. These considerations indicate the existence of parallel, but inconsistently expressed pathways for Zn^{2+} absorption, even if the dominant pathway through ZIP4 is closed. The ZIP4 gene is also detected throughout most of the gastrointestinal tract, in the kidney and in the yolk-sac of embryos [181]; thus it will be of interest to learn whether more subtle systemic manifestations of disease are present, even in zinc-replete individuals.

3.2.2. *Implications of maternal mutations of Zn^{2+} transport for the gastrointestinal tract of offspring*

A disorder of severe zinc deficiency was recognized in mouse pups nursing from dams homozygous for an autosomal recessive mutation, known as the lethal milk gene [182]. In *lmlm* mice the responsible mutation was identified as a premature truncation mutation in ZnT-4 [183]. Subsequent studies in nursing human mothers have questioned a direct connections between ZnT-4 mutation and milk abnormally low zinc content [184], but have demonstrated connections to mutations and single nucleotide polymorphisms in the ZnT-2 gene [185]. These observations highlight the possibility that maternal disorders of Zn^{2+} homeostasis might lead to alterations in offspring that might appear as "phenotypic." In the offspring of the *lmlm* mouse, the dam does not seem phenotypically distressed and the heterozygous offspring can survive with dietary modification. Similarly, in human babies with the ZIP-4 mutation leading to acrodermatitis enteropathica, the heterozygous parent is not distressed, while zinc deficiencies in the homozygous recessive offspring can be arrested or reversed in offspring by supplementation, confirming that systemic zinc management is not necessarily compromised [9]. A recent report by Murgia et al. [186] provides evidence

of some physiologic compensation for ZnT4 mutations in the offspring, including decreased expression of the importer ZIP4 and increased expression of metallothioneins. These considerations indicate that genetic disorders of zinc transporter expression might become apparent only under certain conditions affected by dietary availability, environmental stress or disease.

3.3 Pathobiology of Zn²⁺ Dysregulation in the Gastrointestinal Tract

3.3.1. Disturbances in Mucosal or Luminal Zn²⁺ due to Malabsorption/ Maldigestion Syndromes

It is predictable, and well established, that illness associated with intestinal maldigestion or malabsorption might lead to alterations in absorption of zinc. Thus, Crohn's Disease is associated with zinc deficiencies [187], attributed in part to reduced intestinal absorption of zinc [188], but possibly to effects of immunosuppressive medications. In one curious clinical study, oral supplementation with zinc also seemed to improve intestinal "leakiness" in patients with clinically quiescent disease [189]. In celiac sprue, deficiencies in zinc are a non-specific marker of malnutrition and respond readily to diet modification, even without supplementation [190]. Although complicated by malnutrition and etiologic considerations, it also appears that chronic calcific pancreatitis is associated with organ-centered and systemic disturbances in zinc homeostasis [191-192]; it is postulated that these deficiencies contribute to oxidant stress in the organ [193].

For many years, it has been recognized that different inflammatory illnesses of the GI tract may be associated with zinc deficiency and that their symptoms may be responsive to oral zinc preparations. In patients with gastritis and chronic peptic ulcer, both associated with inflammation due to *Helicobacter pylori*, zinc deficiencies in serum or tissue have been observed inconsistently [194-195], as compared to consistent deficiencies of body stores for iron [196]. In animal models, however, dietary zinc deficiency is associated with poor recovery from experimental ulceration or different agents that induce gastritis [132-133, 197]. Moreover, administration of a variety of zinc-containing preparations appear to improve healing, both in the experimental and clinical setting [133, 135-136, 138-139]. Similar protection has been observed in experimental animals receiving zinc-containing preparations and undergoing protocols that induce colitis or ulceration of the colon mucosa [198-199]. One important difference between studies that have been reported in stomach and those reported in colon is that, in the latter, protection was observed in response to preparations of Zn²⁺ that were conjugated to L-carnosine ("Poleprazinc") which is postulated to have independent anti-inflammatory properties [200]. In contrast, in the stomach, protective and anti-secretory effects were observed in response to poleprazinc and to simple zinc-containing compounds such as zincsulfate or zinc acetate. These considerations suggest that, in the upper GI tract, zinc has protective properties that are independent of the conjugating anion.

In exploring different dimensions of the connection between zinc and mucosal inflammation in the stomach and colon, it was postulated that disturbances in Zn²⁺ handling might occur within the inflammatory microenvironment of the mucosa, particularly in the presence of luminal bacteria. It is well-recognized that thiol-directed oxidants such as monochloramine (NH₂Cl) can be generated continuously by the interaction of ammonia (NH₃), produced by bacterial metabolism, and hypochlorous

acid (HOCl), produced by the myeloperoxidase system of neutrophils [201-203]. Thus, in different *in vitro* models of functionally intact colon crypts or gastric glands, exposure to monochloramine elicits accumulation of Zn²⁺ in the cytoplasm from the sub-nanomolar range under baseline conditions, into the high nanomolar range during exposure to clinically appropriate concentrations of NH₂Cl (50 μM to 200μM) [45, 204]. Effects of such oxidant-induced increases in [Zn²⁺]_i on intracellular targets of, such as caspase-3, were abrogated by pre-treatment with metal chelators such as TPEN. In addition, intracellular buffering of these increases with TPEN markedly reduced indices of cell toxicity during exposure to monochloramine [204]. Intracellular accumulation of Zn²⁺ was completely abolished by pre-treatment with thiol-reducing agents such as dithiothreitol or N-acetylcysteine, and arrested by these agents if they were added following the exposure to NH₂Cl [45, 204]. These experimental observations offer a conceptual framework for understanding the pathological relevance of the intracellular accumulation of Zn²⁺ induced by chloramines and suggest that inappropriate or untimely accumulation of Zn²⁺ can contribute significantly to toxicity caused by oxidant stress in inflamed mucosa.

4. Conclusion and Perspectives

This review emphasizes that the gastrointestinal tract is a major focus of Zn²⁺ absorption and secretion, with consequences for systemic management of different pools of zinc in the body. The recent delineation of different families of transporters offers the opportunity to understand how Zn²⁺ is taken up, distributed and secreted by individual cells in each region of the GI tract and to gain insight into the role of Zn²⁺ to signature functions within each region. Much work remains to be done.

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24. Zinc and the Liver

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Abstract. Zinc is an essential trace element exerting important antioxidant, anti-inflammatory, and apoptotic effects. It is critical to a large number of structural proteins, enzymatic processes, and transcription factors. Zinc affects growth and development, and participates in processes such as aging and cancer induction. Zinc deficiency can result in a spectrum of clinical manifestations. The liver is the main organ responsible for the zinc metabolism. On one hand, zinc deficiency affects several liver functions and on the other hand various liver diseases influence the zinc metabolism. Symptoms, such as poor appetite, loss of body hair, altered taste and smell, delayed wound healing, testicular atrophy, immune dysfunction and diminished drug elimination capacity are common in patients with chronic liver diseases, especially cirrhosis. In the present review, data on zinc homeostasis, its implication in the pathogenesis of various liver diseases, and its therapeutic effects are summarized.

Keywords. liver, liver diseases, therapy, zinc, zinc deficiency

Introduction

Zinc (Zn) plays a key role in numerous biochemical and physiological processes. It is an essential component of more than 150 different enzymes and owes its catalytic effect to its direct involvement in substrate conversion and the stabilisation of enzyme structure. Zn has a structural effect on transcription factors and a regulatory effect on hormones, hormone receptors and gene expression. It also has a proven influence on metabolism, growth and development [1]. Zn plays an important role as a second messenger and signal molecule, and is crucial to the redox process. The cell-damaging oxidative stress that can be the result of a zinc deficiency is a fundamental principle [1]. Although Zn is a redox-inert molecule, it has important antioxidant properties [2]. The protection through the antagonism of redox-active transition metals like copper (Cu) and iron (Fe), and the protection of protein sulphhydryl groups from oxidative damage range among the most important antioxidant properties of Zn [3].

1. General Aspects on the Interaction between Zinc and the Liver

As the main organ involved in zinc metabolism, the liver plays an important role in maintaining zinc homeostasis by acting as a rapidly exchanging repository for zinc

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storage. Liver disease impacts zinc levels and, in turn, is influenced by zinc deficiency [4]. Zn is present in the liver in two forms: one that can be quickly mobilised, and one that can only be mobilised slowly. After absorption by the gut, zinc is rapidly carried to the liver. The regulatory processes are predominantly controlled by hormones such as insulin, glucagon and glucocorticoids [5]. Depending on the specific situation, these substances may lead to a transient dysregulation of the zinc metabolism in the liver cells, with subsequent plasma zinc deficiency. Mediator substances, such as cytokines, have a similar effect.

The intracellular Zn metabolism is regulated by proteins that are necessary for the uptake, the intracellular trafficking and the compartmentalisation of Zn. ZIP (Zrt-, Irt-like protein) proteins are involved in the uptake of Zn, while Zn transporters (ZnT) are responsible for the intracellular trafficking of Zn [6] (for further details see chapter 8). The intracellular compartmentalisation of Zn is achieved via binding to metalloproteins such as metallothioneins (MT). There is a close interaction between MT, an acute-phase protein, and zinc. On the one hand, MT plays an important role in the resorption, distribution and cellular accumulation of zinc, on the other hand, increased zinc intake leads to increased MT synthesis [7, 8]. Zn regulates MT production by binding directly and reversibly to the Zn finger region of the metal transcription factor -1 (MTF-1). This protein then adopts a DNA-binding conformation and translocates into the nucleus, where it binds to metal-response elements of certain genes involved in Zn homeostasis [9]. Increased MT levels enhance the endogenous Zn reservoir, enabling zinc to control oxidative stress [10] (for further details see chapter 4).

There is also a proven, close interaction between zinc and interleukin (IL)-6, an important proinflammatory cytokine for the regulation of the acute-phase gene [11]. The action of zinc in the hepatic synthesis of acute-phase proteins, in the regulation of gluconeogenesis, in controlling reactive substances (e.g. nitrogen oxide) or hydrophilic radicals, and in controlling microbiological growth, are all functions of IL-6 [12]. IL-6 regulates the zinc transporter Zip 14 in the liver, thus contributing to hypozincemia in the acute-phase reaction [13]. Studies using tracers have shown that a complete zinc exchange in the hepatocytes takes less than two days [14]. A reduction of zinc levels in the liver leads to a reduced regenerative capacity of hepatocytes and is associated with impaired liver function [15]. Almost 90% of the intracellular Zn binds to proteins so that the number of free or loosely bound pools of intracellular Zn is very small. Nevertheless, labile Zn regulates important Zn-dependent processes, such as signal transduction, apoptosis and neurotransmission [16]. Changes in zinc status directly alter gene expression. The mRNA levels of MT, cholecystokinin, uroguanylin, endothelin and retinol-binding proteins are affected by changes in zinc status [15].

It can therefore be concluded that zinc deficiency has an effect on hepatic function. The importance of the liver means that zinc plays a key role in numerous biochemical and physiological processes [17]. Zinc deficiency impairs the control of cytochrome P 450 by the thyroid [17, 18].

2. Zinc Deficiency and Liver Disease

The essential importance of zinc for humans was first documented by Prasad et al. in the 1960s [19, 20]. Zinc deficiency in humans is common throughout the world, and it is especially prevalent in areas where the population subsists on cereal proteins. Prasad stated that conditioned zinc deficiency is found in many diseases [20].

In 1956, Bartholomay et al. [21] stimulated interest in the role of the zinc metabolism in liver disease by observing hypozincemia in severe alcoholic cirrhosis. Their findings have since been corroborated by many authors [22, 23]. Zinc concentrations have been also reported to be depressed in white blood cells, pancreatic juice, and in liver tissue in various forms of liver disease [24, 25]. The degree of zinc deficiency depends on the type and severity of liver disease, with the lowest serum levels observed in alcoholic liver cirrhosis with coma [26]. Reduced zinc levels have also been found in patients with acute and chronic viral hepatitis [27]. Cesur et al. reported contradicting results [28, 29]. No significant alterations in trace element concentrations were found in the serum of patients with chronic hepatitis B respectively C compared with healthy subjects. Viral infections produce severe oxidative stress, resulting in cellular damage [27]. Low serum zinc contributes to this oxidative stress [2]. Decreased zinc levels in the liver are associated with impaired liver function and regeneration.

2.1. Causes of Zinc Deficiency in Liver Disease

Zinc deficiency or an altered zinc metabolism in patients with liver disease is caused by a variety of factors, such as inadequate intake, changes in the protein and amino acid metabolism, diminished hepatic extraction, portosystemic shunts, alcohol-induced impaired absorption, and the effect of cytokines, mainly IL-6 [24, 31, 32].

In patients with cirrhosis and ascites, the use of diuretics can lead to increased urinary loss of zinc, reduced circulating albumin levels, and reduced binding of zinc to albumin [32]. Changes in the hepatic MT concentration in patients with liver disease may affect the hepatic binding of zinc. Narkewicz [33] postulated the existence of a regulatory factor involved in zinc homeostasis in the liver, which alters the metabolic or inflammatory milieu of chronic liver disease and induces inappropriate zinc uresis by other poorly defined mechanisms.

2.2. Manifestations of Zinc Deficiency in Liver Disease

Many of the clinical features of liver cirrhosis have been linked to zinc deficiency, including loss of body hair, testicular atrophy, cerebral dysfunction, poor appetite, immune dysfunction, altered taste and smell, reduced vitamin A and thyroid hormone metabolism, altered protein metabolism, delayed wound healing and diminished drug elimination capacity [30, 34, 35, 36]. There is a wide range of possible pathomechanisms of zinc deficiency in liver cirrhosis. Zinc deficiency can lead to oxidative tissue damage and/or the modulation of selected signalling cascades in the liver [37]. Also, zinc deficiency may induce oxidative stress [3] and subsequent conditions such as vulnerability to hepatitis, loss of acute-phase response protection against hepatitis and lipid oxidation. By altering the redox state, zinc deficiency compromises the functioning of oxidative-sensitive transcription factors that can affect cell function, proliferation and survival [38, 39].

2.3. Zinc Deficiency in Liver Cirrhosis and its Complications

2.3.1. General Aspects of Liver Cirrhosis

Liver cirrhosis is a slowly progressive disease, causing scarring and nodularity as a result of chronic injury from a variety of causes. This process distorts the normal liver architecture, interferes with blood flow through the liver, and disrupts its biochemical functions. Unlike in Europe and the United States, where alcohol consumption is the main cause of cirrhosis, the virus-related liver diseases hepatitis B and C are the most common causes of cirrhosis in Africa and Asia.

Clinical manifestations include jaundice, teleangiectasia, splenomegaly, ascites, palmar erythema, scarce pubic and axillary hair, and encephalopathy. Laboratory data may show thrombocytopenia, hypoalbuminemia, prolonged thromboplastine time, and decreased serum zinc in 75 – 80% of patients. Serious complications from cirrhosis, which may lead to hospitalization or even death, include variceal bleeding, ascites, spontaneous bacterial peritonitis, hepatic encephalopathy, hepatocellular carcinoma, hepatorenal and hepatopulmonary syndrome.

The five-year survival rate is 50% for patients with decompensated cirrhosis (portal and metabolic insufficiency) versus 91% without [40, 41].

2.3.2. Ascites and Poor Nutritional State

Ascites is the major cause of complications in cirrhosis patients; approximately 50% of patients with decompensated cirrhosis develop ascites in the course of ten years of being under observation [41, 42, 43]. The presence of ascites indicates an advanced state of liver disease and has important prognostic significance: 50% of patients with ascites can be expected to die within 2 years of the onset [41]. The prognosis for patients with ascites in alcoholic liver disease is considered to be worse than for those with viral liver disease. Ascites is a cause of renal retention of salt and water and resulting portal hypertension leading to the localisation of this excess into the peritoneal cavity, by the activation of neurohumoral responses and by hypoalbuminemia [42, 44]. The liver plays a central role in the regulation of nutrition by controlling the metabolism of macro- and micronutrients, their distribution and appropriate use. Consequently, protein-energy malnutrition is common in patients with advanced liver disease, and constitutes a significant prognostic factor affecting survival, success of a liver transplantation and quality of life [45, 46, 47]. In addition to the disturbed amino acids and protein metabolism accompanied by accelerated skeletal muscle breakdown and impaired ammonia removal in the liver and muscles, patients with cirrhosis suffer from glucose and fat metabolism disturbances with increased energy expenditure, and often develop a hypermetabolic state [46, 47]. In general, the therapy of ascites aims at creating a negative sodium and water balance by restricting the intake of liquid and diuretic agents and, if necessary, by paracentesis with albumin infusions. Recently, several authors reported that the supplementation of branched amino acids alone, or in combination with zinc, may contribute to a reduction in hypoalbuminemia and ascites through an increased supply of substrate for protein and the stimulation of protein synthesis [44, 45, 48].

The ability of branched-chain amino acids (BCAA)-rich supplements to reduce hypoalbuminemia is based on two mechanisms: The first one is the increased supply of substrate for protein synthesis causing albumin synthesis. The second mechanism is the facilitation of protein synthesis with BCAA through the stimulation of the initiation of

albumin mRNA translation via activation of intracellular signal transduction systems [44, 49, 50]. These substrates seem to complement each other: zinc is very important in protein synthesis [51] and albumin is the main medium for zinc transport in the plasma [52].

2.3.3. Hepatic Encephalopathy

Hepatic encephalopathy (HE) is a potentially reversible neuropsychiatric syndrome occurring in both acute and chronic liver disease. While the pathogenesis of HE is still unclear, a variety of mechanisms have been implied [53, 54].

Ammonia plays a key role in the pathogenesis of HE. It induces astrocyte swelling and/or sensitizing astrocytes to swelling by precipitating rather heterogenous factors and conditions. Astrocyte swelling has been shown to impair neuronal neurotransmission and the rate of brain energy production. Astrocyte swelling may also affect the function of important brain proteins by causing oxidative and nitrosative stress with evidence of protein nitration and, as has been more recently revealed, ribonucleic acid (RNA) oxidation [54, 55]. Factors which may induce astrocyte swelling are infections with endotoxins, proinflammatory cytokines and neutrophils impaired by ammonia [54, 56]. Patients with cirrhosis are prone to developing infections, which complicates their clinical course and frequently leads to organ failure and death [57]. Patients with cirrhosis are functionally immunosuppressed and have an impaired host defence mechanisms. A number of abnormalities indicating zinc deficiency have been reported in patients with chronic liver disease. Poor zinc status impairs the nitrogen metabolism by reducing the activity of urea cycle enzymes in the liver [58, 59] and glutamine synthetase in the muscles [32, 60]. Zinc deficiency in association with altered nitrogen metabolism occurs both in experimental models of cirrhosis of the rat [59] and in patients with advanced liver disease [61]. It has been postulated that zinc deficiency may play a role in the pathogenesis of HE, as serum zinc concentrations in patients with this condition correlate inversely with their blood ammonia concentration [26].

A small number of controlled studies have been undertaken to examine the efficacy of zinc in the treatment of HE in cirrhosis patients. The results are conflicting. Katayama observed a synergistic effect of zinc with BCAA and lactulose treatment [62]. Despite these mixed results, zinc supplementation is recommended for HE-patients who do not respond to standard therapy [63]. The ameliorative effect of zinc supplementation on neurological symptoms in patients with liver disease is mediated by peripheral mechanisms. These may involve lowering of blood ammonia levels (urea synthesis, glutamine synthesis in the liver, glutamine synthesis in the muscle). Hypothetically, zinc may influence the blood-brain permeability. It protects against damage caused by free radicals and regulates opioid action in the brain (see figure 1), [64]. Recently, RNA oxidation and an increase of free intracellular zinc (Zn^{2+}) were identified as further consequences of astrocyte swelling and ROS/RNOS production. An elevated Zn^{2+} level mediates mRNA expression of metallothionein and the peripheral benzodiazepine receptors (PBR) induced by hypoosmotic astrocyte swelling. Zn^{2+} also mediates RNA oxidation in astrocytes treated with ammonia. RNA oxidation may impair postsynaptic protein synthesis, which is critical for learning and memory consolidation [55, 65]. This suggests a hitherto unknown role of zinc in signal transduction in HE and cerebral ammonia toxicity. [55]. In astrocyte cultures, hypoosmolarity induces a rapid, transient and reversible Zn^{2+} release. This zinc release

in astrocytes depends on Ca^{2+} -dependent enzymatic NO-synthesis. Ammonia and tumor necrosis factor (TNF)- α both induce astrocyte swelling and, like diazepam, they both induce intracellular Zn^{2+} release in astrocytes [65]. Studies investigating a possible release of Zn^{2+} during HE must still be performed [66].

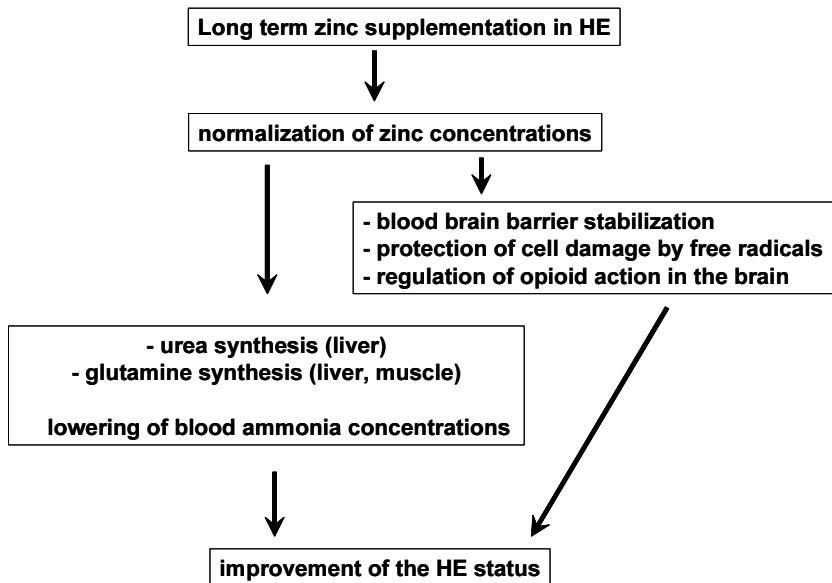


Figure 1. Schematic presentation of the hypothetical role of zinc deficiency in the pathogenesis of HE (modified from [64]).

2.3.4. Hepatocellular Carcinoma (HCC)

HCC is the most frequent primary liver cancer and the most severe complication of chronic liver disease. The annual number of new cases worldwide is approximately 550.000 representing more than 5% of human cancers. It is the third leading cause of cancer-related death [67]. Major risk factors for HCC are well-known and depend on the geographical area. Although many of the specific etiologies that lead to HCC are well-known, the underlying molecular mechanisms leading to hepatocarcinogenesis have not been fully identified. So far, it has not been possible to correlate specific changes in gene expression patterns with HCC development [68]. Nevertheless, cirrhosis, no matter what its cause, remains one of the most significant known risk factor for HCC. HCC may, however, also develop in non-cirrhotic livers. In particular, hepatitis B virus infections have been identified as a cause of HCC in the non-cirrhotic liver and HBV can have a direct oncogenic effect. In these cases, the integration of the viral genome into the host genome causes HCC [69]. Most recently, Yeh et al. [70] demonstrated that HCC can occur as a result of chronic hepatitis C infection even without cirrhosis. They concluded that, even though in some cases, an established case of cirrhosis may have regressed, the possibility that HCV infection and inflammation may be directly oncogenic cannot be excluded. Growing evidence suggests that Non-Alcoholic Fatty Liver Disease (NAFLD) is a relevant risk factor for HCC development [71]. In all etiologies, the hepatocarcinogenesis must involve several indirect

mechanisms including the interplay between chronic inflammation, steatosis, fibrosis, oxidative stress, adipokine/cytokine interplay, which favour cellular growth, and DNA damage.

2.3.5. What Role does Zinc Play in this Process?

Zinc and copper have been reported in a distorted manner in HCC cases, and increased copper levels have been correlated with HCC induction [72]. The zinc content in HCC-tissue was lower than in the surrounding liver parenchyma, and decreased in poorly differentiated HCC. Itoh et al. [73] reported that the levels of copper and zinc measured by electron microscopy were higher in well-differentiated HCC than in moderately or poorly differentiated HCC. The zinc content was not related to the presence or absence of HCC. Serum zinc levels were either increased or unaffected [6] in cirrhotic patients with HCC, compared with those in cirrhotic patients without HCC. From a group of patients with HCC ($n = 73$), 35 patients (47%) had decreased serum zinc levels. While most patients with chronic hepatitis B (4 out of 4 patients) and chronic hepatitis C (19 out of 20 patients) showed decreased serum levels, from the group of patients with alcoholic liver cirrhosis ($n = 27$) 14 had decreased (51.9%) serum levels and 13 (48.1%) had normal serum zinc levels (range: 11.00 – 23.00 $\mu\text{mol/l}$); (unpublished results). There is considerable evidence to suggest that sex hormones play a role in the development of HCC [74]. A disturbance of the sex hormone metabolism is common in cirrhosis. In an animal model of HCC, Frezza et al. [74] found increased serum levels of oestrogen, zinc and copper. The authors suggested two different hypotheses concerning HCC carcinogenesis: 1. When HCC develops in an animal, the hepatocellular metabolism is disturbed more than if cirrhosis occurs alone. This leads to increased levels of oestrogen, zinc and copper. 2. The cancer rate is higher in animals with increased levels of oestradiol, zinc and copper. Ebara et al. [72] reported that in HCV-positive patients, an increased copper level in cirrhotic tissue was the only predictive factor for HCC development. It was also suggested that zinc deficiency may play a role in the pathogenesis of HCC by controlling the redox state in the cell, DNA and RNA synthesis [75]. Zinc deficiency enhances oxidative stress by reducing MT and copper/zinc-SOD levels. In HCV-positive cirrhotic women with HCC, liver copper/zinc-SOD activity was reduced, while the MDA level was increased [6]. For further principles of the role of zinc in the development of cancer see chapter 14.

2.3.6. Liver Cirrhosis and “Liver” Diabetes Mellitus are linked to Zinc Deficiency

The association between liver cirrhosis and variations of glucose tolerance ranging from postprandial hyperglycaemia to clinically overt, nonketotic, non-insulin-dependent diabetes mellitus (type 2 diabetes mellitus) has been extensively documented and discussed [76]. The liver is affected by diabetes largely as a consequence of altered metabolic processes and drug-induced hepatotoxicity. Conversely liver disease, and cirrhosis in particular, is associated with a disorder of the glucose metabolism. The mechanisms of glucose intolerance in chronic liver disease have not been fully identified, but could be due to one or several factors, such as glycolytic enzyme activity, changes in specific glucose transporter expression, reduced insulin production, or impairment of the membrane receptors for insulin [77, 78]. Poor zinc status is common in both liver cirrhosis and diabetes mellitus. As shown above, multiple mechanisms underlie zinc deficiency or altered zinc metabolism in patients with liver disease (see figure 2). Zinc plays a specific role in nutrition. Many of the

clinical features of liver cirrhosis and diabetes mellitus have been linked to zinc deficiency (see above). The urea cycle enzyme, ornithine carbamoyltransferase in the liver, and glutamine synthetase in the liver and muscles, are zinc-dependent [58]. Zinc plays an important role in protection against damage by free radicals. Zinc is an integral part of the insulin molecule and crucial for the synthesis, storage, and secretion of insulin in pancreatic islet cells [79]. It is important for the receptor and postreceptor function of insulin [78]. Zinc supplementation improved neurological symptoms and signs of malnutrition in patients with liver cirrhosis and hepatic encephalopathy with and without diabetes mellitus [64, 80]. In conclusion, zinc deficiency may be the link between liver cirrhosis and "liver" diabetes mellitus (see figure 2; [81]).

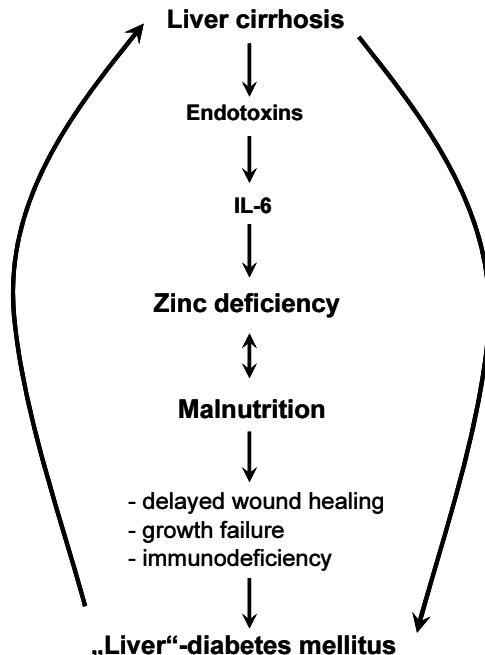


Figure 2. Hypothetical model of the link between liver cirrhosis and "liver" diabetes mellitus (modified from [81]).

3. Zinc in Hepatitis B and C Virus Infections

3.1. General Aspects

The hepatitis B virus (HBV) and hepatitis C virus (HCV) affect worldwide over 300 and 700 million people, respectively. Both HBV, a DNA virus, and HCV, a RNA virus, are hepatotropic, and both frequently lead to hepatitis and, as a consequence, to potentially fatal complications such as hepatocellular carcinoma. A high proportion of patients infected with HBV (5 – 10%) and HCV (70 – 80%) develop chronic infections characterized by absent, weak or narrowly focused T cell responses [82]. It is likely that early immune avoidance mechanisms contribute to the T cell responses in combination with various other strategies [83, 84, 85]. Even though the two viruses differ considerably in terms of their interaction with the host immune system, all

current treatment protocols aimed at clearing either of the viruses from the body use interferon (IFN)- α . This implies that the innate immune system is of pivotal importance for the development and maintenance of chronic infections versus viral clearance [84]. Viral infections produce severe oxidative stress and secondary cellular damage of varying severity.

Zinc ions are crucial for the immune system in a variety of aspects, including the normal development, differentiation and function of cells belonging to both innate and acquired immunity [86] (for further information see chapters 10-13). Among the immune cells that are affected by zinc deficiency, T lymphocytes are noted to have the highest susceptibility. Zinc deficiency causes substantial impairment of cellular immunity, oxidation, and damage to DNA.

3.2. Zinc in the Pathogenesis of Chronic Hepatitis C

The immune responses of patients with resolved infections are often significantly different from those with chronic viraemia. The majority of patients with acute resolving HCV infections lack neutralizing antibodies and patients with a chronic course of infections develop neutralizing antibodies only after viral persistence has been established. In contrast to acute resolving HCV infection, T cell responses are weaker and only nonspecific or even absent in persistent acute HCV infection.

The imbalance between cellular and humoral immunity in chronic hepatitis C is partly due to an imbalance between Th1 and Th2 cytokines. Th1 cytokines (IL-2, IFN- γ , TNF- α) support cellular effector mechanisms, whereas Th2 cytokines (IL-4; IL-10) support the production of antibodies and inhibit cellular mechanisms. Ultimately, an insufficient T cell immune response can be seen as the main cause for the chronification of HCV infections [87]. Recent studies have shown that endogenous factors such as regulatory T cells (Treg), immune suppressive cytokines and inhibiting receptors help to restrict the virus-specific T cell response, as is the case in chronic HBV and HCV infection [83, 85]. This reflects the efforts of the infected organism to protect itself against immune-mediated pathological changes.

Factors of this type, which lead to a functional modulation of monocytes and macrophages over the course of a chronic HCV infection, include IFN- γ , endotoxin (lipopolysaccharide, LPS) and the HCV core protein [85]. A key role in the innate immune system is played by toll-like receptors (TLR). These receptors can recognise not only bacterial lipopolysaccharides and lipoproteins, but also viral antigens and nucleic acid. Not only do they stimulate unspecific immune mechanisms, but they are also important for the production of specific immune reactions and the immunological memory.

Dendritic cells (DC), which synthesise TLR, can activate T lymphocytes. TLR's are also closely involved in zinc homeostasis and the activation of DC, which play an important role in the clearance of pathogenic structures [88]. Stimulation with the TLR4 agonist LPS restricts the expression of zinc transporters into the DC, which in turn leads to a reduction of free intracellular zinc. Zinc plays an important role in cellular and humoral immunity. Zinc influences T and B lymphocytes as well as NK cells and monocytes [89, 90, 91]. It performs special tasks in the antigen-specific immune response (T lymphocyte-dependent cellular immunity and antibody response via antigen-stimulated B lymphocytes) and in unspecific immune mechanisms (phagocytosis, complement activation), which cannot be performed by any other trace element and which give zinc a certain exclusivity. In this way, zinc influences, among

other things, the binding of the signal transduction element p56^{lck} to CD4+ or CD8+ cells and is therefore essential for T cell activation [92]. By binding to the NK cell receptor ‘killer cell-inhibitory D receptor (KIR2D)’, zinc regulates the activity of NK cells [93]. Zinc deficiency impairs the immune response, and many diseases are accompanied by secondary zinc deficiency that aggravates the disease. Zinc deficiency has, above all, an adverse effect on T cells by reducing their number and impairing their function. This includes a shift in the Th cell response to Th2 predominance [86]. Considering the essential role of zinc for effective T cell function, zinc deficiency may be significant for the pathogenesis of chronic hepatitis C, and possibly also for the success of antiviral treatment.

The importance attributed to the role that the essential trace element zinc plays in the immune system, has been reconsidered in recent years. Although its function as a structural component of many enzymes has been known for decades, current experimental evidence points to an additional function of free or loosely bound zinc ions as an intracellular signal transmitter [94].

3.3. Zinc in the Treatment of Chronic Hepatitis C

Current standard treatment of chronic HCV, consisting of pegylated interferon (PEG-IFN) and ribavirin, is associated with a wide range of side effects, contraindications and costs, and achieves viral clearance in just 50–55% (genotype 1) to 80% (genotype 3) of cases. Ribavirin is an essential ingredient in the treatment of chronic HCV infection and required to achieve a sustained virological response. It is a weak antiviral agent and does not cause reduction in serum HCV-viruslast when used alone. This situation makes the development of more efficient treatment regimes with fewer side effects and costs a top priority. In our own experience, 40% of patients with chronic hepatitis C have a zinc deficiency which is aggravated during antiviral treatment. Up to 75% of patients with liver cirrhosis are at least temporarily deficient in zinc [95]. Many years of observation have shown that serum zinc concentrations decrease in the course of treatment of chronic hepatitis C with PEG-IFN and ribavirin, especially in the case of responders. This could be explained by the increased zinc consumption in cytokine-induced immunological processes. This explanation is supported by the fact that IL-6 plasma concentrations, which are raised at the beginning of the treatment, normalise towards the end of the course of treatment. Zinc levels can be normalised by the temporary administration of a zinc supplement. The symptoms which accompany antiviral treatment, such as hair loss, dry skin and brittle nails (symptoms of zinc deficiency), can be partially or entirely alleviated by administering zinc [96]. These observations, made on the basis of own experience of individual cases, were confirmed by the results of a randomised study by Takagi et al. [97], which showed a higher response rate for a combination of IFN- α plus zinc compared to IFN- α alone.

Alongside the insufficient T cell functions, increasing importance is being attached to the presence and action of oxidative stress in patients with HCV as a factor leading to chronification of the disease [98, 99]. It is assumed that the hepatic oxidative stress induced by the HCV is a direct consequence of the virally triggered destruction of mitochondria and other cell organelles of the hepatocytes, on the one hand, and leads to the initiation of an inflammatory reaction in the liver, on the other [98, 100]. Zinc plays an important role in the redox process as a signal molecule and second messenger. Disruption of zinc homeostasis can contribute to the chronification of various diseases. Cell-damaging oxidative stress, which can be induced by zinc deficiency, is a

fundamental principle [101]. Studies by Murakami et al. [102] showed that zinc substitution in patients with chronic hepatitis C leads to the induction of antioxidative functions in the liver, and to decreased liver damage during treatment with PEG-IFN and ribavirin. Himoto et al. [103] recommend the administration of zinc supplements for patients with chronic hepatitis C, as zinc significantly reduces the disease activity by lowering iron concentration. It has not yet been possible to conclusively assess the role of nuclear factor (NF)-kB in the persistence of a chronic HBV or HCV infection, as there are as yet no reliable data from primates or humans [104]. Irrespective of this, zinc substitution has been proven to lead to the reduction of inflammatory cytokines as it inhibits I kB phosphorylation and NF-kB activation [105]. Recent studies by Yuasa et al. [106] have shown that zinc substitution negatively influences HCV replication. The mechanisms, underlying this inhibiting influence on the HCV genome in HCV RNA-replicating cells, have not yet been clearly identified. Clinical observations of the positive effect of zinc substitution on a large proportion of patients receiving treatment for chronic hepatitis C, should therefore be confirmed by experimental studies. The proven and assumed effects of zinc in the treatment of chronic viral infections, including hepatitis C, are produced via immunological reactions, antiviral defence mechanisms and the role of zinc as an antioxidant (see figure 3), [107, 108].

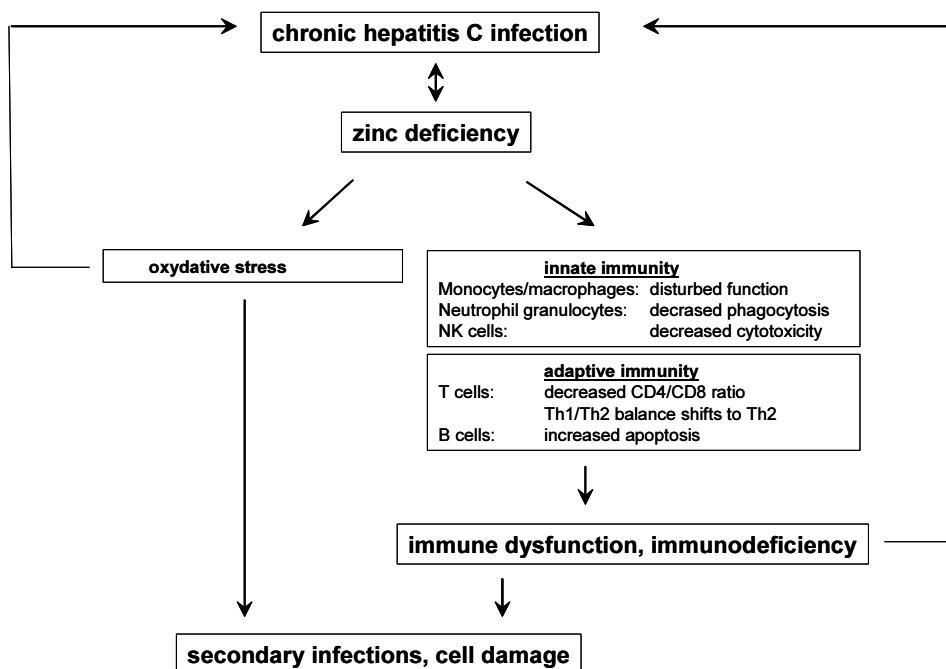


Figure 3. Interaction between zinc homeostasis and chronic hepatitis C infection (modified from [108]).

3.4. Hepatoprotective Effects of Zinc

Experimental studies have shown that zinc has a hepatoprotective effect against a variety of chemical substances such as tetrachloromethane, thioacetamide, bromobenzene,

acetaminophen and various metals. These studies, some of which go back more than 30 years, characterised the effects of zinc as anti-fibrotic and anti-inflammatory [109].

Additional research efforts over the past decade have contributed to new insights into the hepatoprotective effects of this trace element. Souza et al. [110] found that zinc protects stellate cells from cytotoxic damage through cadmium.

Scientists have explained that this is thanks to the membrane-stabilising effect of zinc on the one hand, and the maintenance of the intracellular redox balance on the other. Zhou et al. [111] and Sakaguchi et al. [112] provided similar explanations, stating that the protective effect of zinc against thermal damage or endotoxins, i.e. oxidative stress, can be explained by its anti-oxidative qualities in interaction with metallothionein. Contrary to these findings, Krones et al. [113] were unable to show any protective effects of zinc in experimentally triggered acute endotoxaemia. Quite to the contrary: when lipopolysaccharides were infused simultaneously, the reinforced synthesis of proinflammatory cytokines, particularly TNF- α and IL-6, led to deterioration. For this reason, the authors advise against the prophylactic administration of zinc in cases of acute sepsis. Oral administration of zinc salts led to a significant reduction of serum bilirubin levels in hyperbilirubinaemic rats, which may be due to the inhibition of the enterohepatic bilirubin cycle [114]. In conformity with Mendez-Sanchez et al. [115] the authors recommend oral zinc therapy in newborn jaundice, Crigler-Najjar syndrome and for the prevention of pigment gallstones in predisposed patients. Zinc has also been described as having a hepatoprotective effect in cases of lithium poisoning [116] and halothane intoxication [117]. Current animal studies on the influence of zinc on genetic expression by Liu et al. [118] found that non-toxic, hepatoprotective doses of zinc lead to consistent gene expression including a dramatic upregulation of MT, moderate activation of (NF-E2-related factor 2) and acute-phase genes, and a moderate suppression of metabolic enzymes. According to the authors, this behaviour of genetic expression plays an integral role in the zinc-induced protection against various hepatic toxins.

3.5. Alcohol-Induced Hepatic Damage

In rats which had been pre-treated with alcohol, zinc administration led to an increase in alcohol dehydrogenase in the stomach, in addition to effects on prolyl hydroxylase and lipid peroxidation in the liver. This explains the positive effects of this trace element on chronic alcoholics [119]. Lambert et al. [120] found that pretreatment with zinc clearly limited the extent of acute alcohol-related liver damage. They explained this effect with zinc's inhibition of the disruptive effect of alcohol on the permeability of the small intestine. Alcohol consumption induces apoptosis in various tissues including liver and lymphatic tissue. According to Szuster-Ciesiealski et al. [121], the increased apoptosis in the PBMC (peripheral blood mononuclear cells) of patients with cirrhosis can be arrested by administering zinc. Zinc mainly inhibits the mitochondrial pathway of immune cell apoptosis. Chronic alcohol consumption also leads to bone changes, primarily osteoporosis. As well as a direct action on bone synthesis, many effects contribute to impairing the bone metabolism via the accompanying malnutrition, alcoholic myopathy, hypogonadism, hypercorticism and liver damage per se [122]. In experiments using mice, Zhou et al. [123] were able to show that zinc supplementation after alcohol consumption prevented a fall in the zinc content of the liver and acute liver cell damage. This hepatoprotective effect of zinc is thought to be due to its antioxidant properties. Although the process is multifactorial, the main action of zinc is

thought to be due to its inhibitory effect on the cytochrome P450 system (CYP2E1) and to its antioxidant properties. These findings demonstrate the therapeutic potential of zinc for preventing and treating alcoholic liver damage. According to recent research by Kang et al. [124], zinc supplementation leads to a reduction in alcoholic liver damage by alleviating oxidative stress and TNF- α -mediated hepatocyte damage, by increasing liver regeneration and by suppressing the death receptor mediated pathway.

The following biological actions of zinc can explain its hepatoprotective effects:

1. Zinc stabilises cell membranes and inhibits lipid peroxidation
2. Zinc induces metallothionein synthesis
3. Zinc diminishes the effects of cytochrome P450
4. Zinc improves the protein synthesis function of the liver
5. Zinc stabilises the intestinal permeability

3.6. Wilson's Disease

Wilson's disease is an hereditary disorder of the copper metabolism which leads to copper being stored in various organs including the liver and the brain. The aim of treatment is to reduce the amount of copper stored by achieving a negative copper balance. At present four drugs are used as copper-eliminating substances in Wilson's disease: the copper chelating agents penicillamine, trientine and tetrathiomolybdate, the last of which forms a tripartite complex with copper and protein, and zinc. Treatment with zinc was introduced by Schouwink [125] in 1961. Reports have repeatedly been published citing intermittent administration of zinc as a useful alternative to penicillamine. Metabolic studies [126, 127] have provided evidence that the regular oral administration of a zinc preparation is needed to block the absorption of copper in the gut. Ferenci [128] recommends oral zinc as an initial treatment in the preclinical stage of Wilson's disease and in patients in whom the disease is manifested through neurological symptoms. According to Brewer et al. [129] zinc and tetrathiomolybdate are the most effective non-toxic drugs for most phases of Wilson's disease. Zinc is particularly appropriate both for maintenance therapy and for the treatment of presymptomatic patients, pregnant women and children [130]. Tetrathiomolybdate is the first-line treatment in patients with neurological symptoms. On the basis of many years' experience, Brewer et al. [126] recommend that zinc therapy for Wilson's disease should consist of 3 x 50 mg zinc/day for adults and 3 x 25 mg zinc/day for children. The serum or plasma zinc concentration and the excretion of copper in the urine should be monitored at intervals of 6-8 weeks. The excretion of copper in the urine should be >125 µg/24 hours. Excessive doses of zinc can lead to copper deficiency. The literature provides many examples of copper deficiency related to excess supplemental zinc, affecting many tissues and functions (see also in "conclusions and future perspectives"). According to investigations by Farinati et al. [131], peroxidative damage takes on considerable significance in Wilson's disease with increased use of reduced glutathion and increased glutathion turnover. Treatment with penicillamine is not sufficient to compensate for the disturbed lipid peroxidation. The authors feel that zinc exerts its crucial action here and that its lowering effect on the copper level is less important. Acute hepatitis following zinc administration was reported by Castilla-Higuero et al. [132] as a rare side effect of zinc therapy in Wilson's disease. Liver function values returned to normal as soon as the patient stopped taking zinc. This is contradicted by the findings of Medici et al. [133], who

investigated the effects of zinc on acute hepatitis that was induced in rats by giving excess copper. The ingestion of zinc prevented acute hepatitis, or at least caused it to take a much milder form. The amounts of metallothionein and zinc in the zinc-treated rats increased significantly while the copper content was significantly reduced.

4. Conclusion and Perspectives

There are existing interesting data on the relationship between zinc and the liver, a lot is yet speculative and further intensive research is needed. The liver is the main organ responsible for the zinc metabolism. Zinc affects the liver in many ways. On the one hand zinc deficiency affects liver functions and on the other hand various liver diseases influence the zinc metabolism. Over the years human and experimental studies have documented reduced serum and liver zinc levels in acute and chronic liver injury and HCC. Moreover zinc deficiency has been implicated in the pathogenesis of these diseases. Symptoms of a zinc deficiency, such as poor appetite, loss of body hair, altered taste and smell, delayed wound healing, testicular atrophy and immune dysfunction are common in many patients with chronic liver diseases, especially cirrhosis. Therefore, serum or plasma concentrations of zinc should be determined in cases showing typical symptoms of zinc deficiency.

Little research provides insight into the optimal doses of zinc in liver disease. No studies of zinc illuminated the doses or dose range at which is both efficacious (for improving or curing the underlying disease) and safe (with acceptable risk of adverse effects) [134]. Clinical trials of zinc supplementation are difficult to perform and experimental studies are difficult to interpret because there are differences in experimental design. To date, there are no reports of systemic zinc toxicity within a range of doses for supplementation, and, in a study of zinc metabolism by Lowe et al. [135] healthy adult males given 65 mg (or about 925 µg/kg) of IV zinc sulfate overnight no adverse effect. The recommendations issued by various committees are guidelines, not precisely defined limits. The toxicology literature indicates that symptoms of zinc toxicity are relatively nonspecific and include vomiting, diarrhea, fever, lethargy, and muscle pain [136].

The maximum of dose for zinc substitution has not yet been clearly defined. However, this is of great importance for long-term supplementation. 100 mg or more of elemental zinc per day leads to severe immunological damage [137, 138]. Supplementation with quantities of zinc above the suggested upper limit can result in copper deficiency, especially if the form of zinc in the supplement is readily bioavailable [139]. Experimental studies have shown that zinc concentrations above 0.5 mmol (about equivalent dose of 45 mg elemental zinc) have a toxic effect on immune cells and inhibit DNA synthesis and cytokine production [91]. According to Prasad [140] oral doses of up to 45 mg elemental zinc per day are not to be considered toxic.

Grüngräff and Reinhold [108] recommend on the basis of more than 15 years experience of zinc substitution in patients with different forms of liver diseases and proven zinc deficiency the following procedure: if the zinc levels in the serum < 10 µmol/l: zinc substitution 15mg/day; if the zinc levels < 8 µmol/l: zinc substitution 30mg/day. The patient should be monitored after 6 – 8 weeks. Once the zinc concentrations have normalized, the zinc administration can be stopped. Monitoring checks should be carried out regularly, depending on the severity of the liver disease: liver cirrhosis – acuity, hepatic encephalopathy, decompensation, nutritional state – and

in cases of chronic hepatitis B or C receiving antiviral therapy. Lasting replenishment of zinc stores can take up to one year.

There is hope that we will have a clinical marker and/or test to analyse the zinc concentrations intracellularly, and that we will be able to identify a zinc deficiency exactly in future.

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25. Zinc and Diabetes

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Abstract. Diabetes is a set of metabolic disorders in which there is a major dysregulation of blood glucose levels. All but a very small number of patients suffer from either type 1 and type 2 diabetes, both having the same long-term complications. There is a longstanding relationship between zinc homeostasis and maintenance of glycaemia. Diabetes affects intracellular zinc homeostasis, alterations of which effects carbohydrate metabolism. Zinc has strong functional interactions with the insulin biosynthesis/secretion machinery, the immune system, and insulin signalling. Zinc deficiency also impairs cellular antioxidant defense. In this chapter we will review the multiple roles of zinc in diabetes, including its importance for insulin-producing cells at the molecular level. The role of zinc transporters in the insulin secretion pathway and effects on beta cell protection are also discussed.

Keywords. Zinc, diabetes, beta cell, insulin, blood glucose, zinc transporter.

Introduction

Diabetes Mellitus represents one of the most common chronic diseases and is a set of chronic metabolic disorders in which there is a major dysregulation of blood glucose levels (hyperglycaemia). According to the World Health Organisation (WHO), the current growth rates of diabetes have reached epidemic levels (see <http://www.who.int/research/en/>), affecting 300 million individuals worldwide and expected to rise to 400 million by 2025. Despite treatments currently available diabetes remains a major health problem due to the high vascular burden that relates with residual hyperglycaemia and associated metabolic disorders. The disease is among the leading cause of blindness, end stage renal disease and non-traumatic limb amputation in adults. Diabetes has become one of the major causes of premature illness and death in most countries, mainly through the increased risk of cardiovascular disease (CVD) [1]. Indeed, the incidence of premature death from diabetes is similar to that of HIV/AIDS, yet the problem is largely unrecognized.

Insulin, produced by beta cells in the pancreas, is an essential hormone that stimulates glucose clearance from the blood to muscle, fat and liver cells. The hallmark of Diabetes Mellitus is a loss of control of glycaemia due to a lack of insulin which may be relative or absolute depending on the type of diabetes. Although there are many forms of diabetes, both genetic and environmental factors usually combine to provoke

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the disease. All but a very small number of patients suffer from one of two forms of diabetes:

Type 1 diabetes (T1D), formerly known as Insulin Dependent Diabetes Mellitus, IDDM, accounts for about 5–10% of all cases of diabetes. It is generally caused by the appearance of auto-antibodies, leading to an autoimmune destruction of pancreatic beta cells and resulting in virtually no insulin being produced by the pancreas. It has often been called ‘juvenile’ diabetes, since it is usually first diagnosed primarily children and teenagers; though adults can also develop T1D. Without insulin being produced to allow glucose absorption by target tissues, tissues turn to fats as the main intracellular energy source, with the consequent generation of ketone bodies and organic acids. Without exogenous administration of insulin, the resulting diabetic ketoacidosis may be fatal. Patients suffering from T1D are thus dependent on insulin for life (for review see [2] and [3]).

Type 2 diabetes (T2D, formerly termed Non Insulin Dependent Diabetes Mellitus, NIDDM) is the most common form of the disease, accounting for over 90% of cases. Its classical symptoms are polyuria (frequent urination), polydipsia (increased thirst) and polyphagia (increased hunger). The primary cause of T2D is often considered to be defective insulin signaling, i.e. insulin resistance caused by a combination of genetic and environmental factors. In contrast to T1D, in T2D the pancreatic beta cells initially function correctly and even secrete large amounts of insulin into the blood. However, the released insulin fails to act effectively upon its target organs, e.g. muscle, fat and liver, a condition known as insulin resistance. As a consequence, beta cells are forced to secrete more and more insulin. While insulin resistance usually reaches a steady plateau in the development of T2D, islet beta cell function declines continuously over time, eventually leading to overt T2D [4]. Therefore, insulin resistance by itself is not sufficient to cause the hyperglycaemia characteristic of T2D. The latter only develops after beta cell failure to compensate for the increased demand in insulin (for review see [5] and [6]). Correspondingly the majority of genetic associations that have been identified as affecting T2D risk lead to a decrease in beta cell function [7–10]. Different classes of drug treatments exist, both to attenuate insulin resistance and increase insulin secretion in T2D. However, the progressive nature of the disease means that it requires an escalation of therapeutic efforts over time as metabolic control deteriorates. Unfortunately, most existing treatments are unable to correct the metabolic errors on a chronic basis. Thus, many patients suffering from T2D may ultimately require injection of exogenous insulin as in T1D. Despite their differing etiology, both Type 1 and Type 2 diabetes result in the same principal symptoms, i.e. hyperglycaemia and dyslipidemia; and lead to the same complications.

Whilst it is clear that the common forms of T2D are complex polygenic traits, some rare forms of diabetes, such as “MODY” (Maturity Onset Diabetes of the Young), are monogenic and result from a single mutation in a particular gene (for review see [11]). Monogenic diabetes resulting from mutations that primarily reduce pancreatic beta-cell function accounts for 1–2% of diabetes cases, although affected individuals are often misdiagnosed as having either Type 1 or Type 2 diabetes.

There is a longstanding relationship between zinc homeostasis and maintenance of glycaemia, which is known to be complex. Indeed, it is still unclear whether a disturbance in zinc homeostasis might be the cause or the consequence of dysregulation of glucose homeostasis. There is no doubt, however, that zinc is crucial for the pancreas and the regulation of blood glucose. Early evidence for this view emerged in the 1930s when Scott discovered that the amount of zinc contained in the pancreas of

diabetics patients was only one-half that of non diabetic individuals [12]. At that time, zinc ions were already used in insulin preparations for diabetics to obtain zinc-insulin crystals and thus extend the duration of action of insulin (see below). Since then, zinc has been proved to be an essential trace element, being one of the most abundant and important metal ions in biology. Hence, it affects many physiological functions and binds to more than 10% of all proteins in the proteome; and because of its pleiotropic effects, perturbations of zinc homeostasis can have catastrophic consequences. Zinc is linked to many major patho-physiologies including impairment of brain function, autoimmune disorders, and diabetes (for review see [13]). Recent advances in our understanding of zinc biology have placed this once obscure metal in the center stage, rivaling the biological importance of calcium. In this chapter we will look at the interactions of zinc with diabetes, and particularly at insulin synthesis/secretion and signaling and on the role of zinc at the level of the pancreatic beta cell, with a particular focus on the zinc transporter ZnT8, encoded by SLC30A8, and its influence on both Type1 and Type 2 diabetes.

1. Zinc and Insulin Interactions

As discussed above, insulin is produced and stored in pancreatic beta cells, and is released by exocytosis in response to external stimuli, notably elevated blood glucose concentrations [14]. Insulin is stored in a crystalline form as a zinc:insulin (2:6) complex. Hence, the zinc content of the pancreatic beta-cell is among the highest in the body. The physical-chemical interactions between zinc and insulin have been known for decades. Since the discovery of insulin in the early 1920s by Best and Banting [15], it was clear that addition of zinc to insulin preparations extended the duration of action of insulin. In the 1930s, zinc ions were added to insulin *in vitro* to produce PZI (protamine zinc insulin) and used in clinics. After zinc addition the quantity of insulin necessary to control the blood glucose was significantly reduced, thus requiring fewer injections [16]. More recently, researchers aimed to develop insulin preparations acting with different range of actions, e.g. long- and short-acting insulin preparations. Removing zinc to avoid crystallization and accelerate the onset of insulin action is a route to achieve this goal. Indeed, it has been shown recently that insulin glulisine [3(B)-Lys, 29(B)-Glu-human insulin] has the most rapid onset of action because of its zinc-free formulation, thereby adding flexibility in postprandial blood glucose control [17].

1.1. Zinc and Insulin Biosynthesis

Insulin is initially synthesized in the rough endoplasmic reticulum as a single chain polypeptide, preproinsulin (Figure 1). The N-terminal hydrophobic extension is then rapidly removed, leading to the formation of proinsulin. Interestingly, proinsulin and insulin have roughly the same association behavior, e.g. they can form dimers by spontaneously folding into the correct three-dimensional structure. In the presence of zinc ions, both insulin and proinsulin dimers further aggregates into hexamers containing bound zinc [18]. The most fully occupied zinc site is located at the center of the hexamer and coordinated via HisB10 and GluB13. Additional zinc binding in the 2-zinc insulin crystal takes place on the hexamer's surface, where interactions with glutamic acid and histidine sidechains are principally involved. Hexamer formation has

been shown to be fundamental in the processing of proinsulin to insulin. A coding mutation in the human insulin gene (HB10D) is associated with familial hyperproinsulinemia [19]. HB10D-Insulin is capable of forming dimers, but it does not hexamerize nor crystallize in the presence of zinc, thereby reducing proinsulin processing [20]. It is likely that the insulin moiety in the proinsulin hexamer adopts the same conformation as the insulin hexamer. However, while insulin:zinc complexes tend to precipitate, proinsulin:zinc hexamers remain soluble [21].

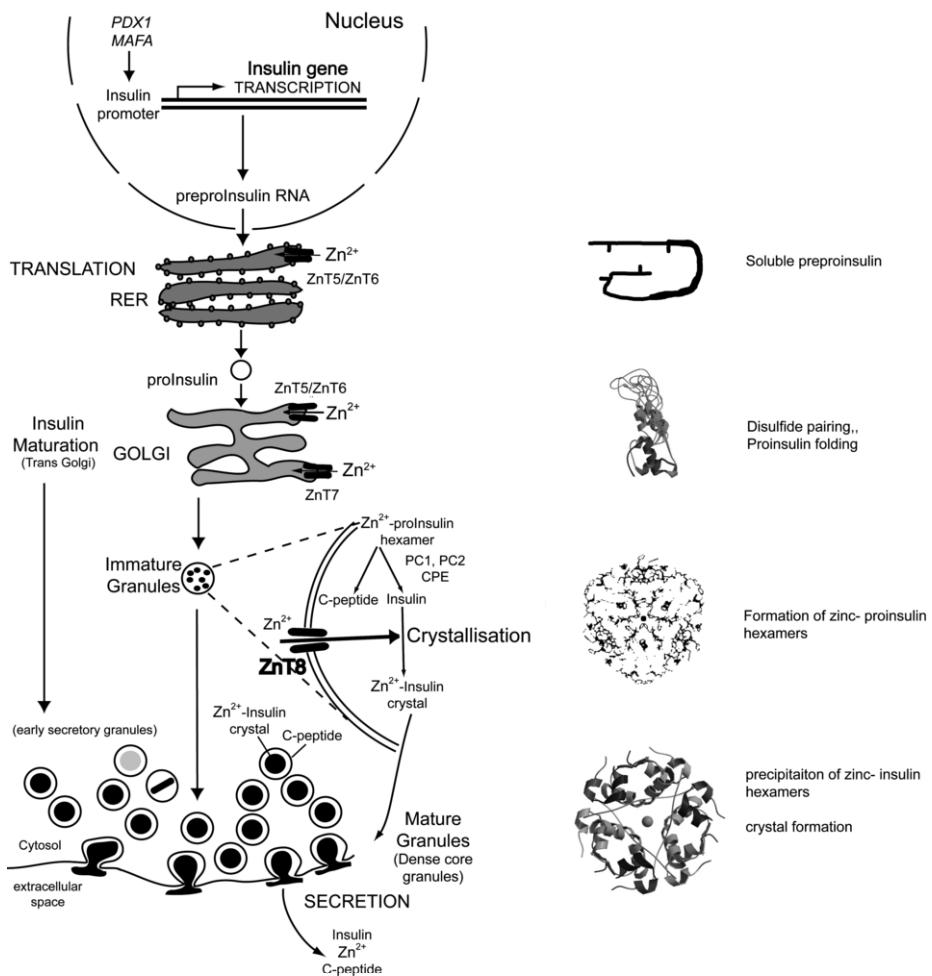


Figure 1 : Insulin biosynthesis. Schematic representation of the cellular (left) and molecular (right) events leading to insulin biosynthesis. See text for details. Adapted with permissions from [28, 29]

NMR Structure of proinsulin : PDB 2KQP ; Zinc-insulin hexamers : PDB 2INS

Hence, proinsulin may remain soluble during its transport to the Golgi apparatus, where prohormone convertase enzymes transform pro-insulin to insulin by removing

C-peptide (for review see [22]). Proteolytic conversion of insulin really starts within the clathrin-coated storage vesicles [23]. In fact, immature, pale secretory “progranules” are formed in and bud from the Golgi. These granules contain proinsulin-zinc hexamers. They are further processed into mature insulin and C-peptide by the prohormone convertases PC1/3 and PC2 [24]. Zinc:proinsulin hexamer formation has been suggested to increase the degree of conversion of soluble pro-insulin to insoluble insulin [25]. The zinc:insulin hexamers then rapidly precipitate to adopt a crystalline form, observed in most animals. Moreover, insulin is an intrinsically amyloidogenic peptide, forming amyloid fibrils; formation of fibrils by insulin requires substantial unfolding of the native protein [26]. Besides being cytotoxic, these large misfolded oligomeric intermediates can also be immunogenic if they occur in the circulation, and might induce autoantibodies against insulin, especially in zinc-deficient conditions. Importantly, it has been shown very recently that zinc ions, co-secreted with insulin, might suppress fibrillization of insulin at its release sites and in the circulation [27]. Hence, besides its paracrine and autocrine functions in islet cells, zinc co-secreted from pancreatic beta cells might also protect the organism from insulin fibrillization and formation of intermediary insulin oligomers/aggregates.

1.2. Insulin Crystallization and Zinc Transport

More recently, the role of zinc in insulin crystallization has been highlighted by studies on zinc transporters, namely ZnT8, a zinc transporter of the SLC30A family. ZnT8 mRNA [30] and protein has been shown to be almost exclusively confined to pancreatic islets [31] and to participate to the regulation of insulin secretion [32]. Interestingly, mice deleted for SLC30A8, the gene encoding for ZnT8, display only “pale” progranules, and do not show the typical pattern of dense core vesicles characteristic of zinc-insulin crystal. The beta cells of KO mice contain only marginal amounts of zinc compared to control cells, and are not stained by dithizone nor Timm’s staining. The “pale” granule present in the KO mice strongly suggested altered insulin crystal condensation [33-35]. Therefore, since there was no compensation by other ZnT proteins, the zinc transporter ZnT8 was suggested to be crucial for both zinc transport in the insulin granules and correct insulin crystallization, which could not occur if (almost) no zinc is present in these vesicles. Strikingly, the importance of zinc for insulin crystallization was already revealed in the late 1960’s, at a time where no zinc transporter was identified. Indeed, Boquist et al. showed that zinc deficiency leaded to decreased beta-cell granulation [36], i.e. decreased insulin crystallization, consistent with the importance of zinc content in the pancreatic beta cells for proper insulin storage. Importantly, insulin biosynthesis is also regulated by other zinc transporters. Indeed, ZnT5 is expressed at the beta cell level, and participates in zinc export to the ER/Golgi system [37], suggesting that this transporter contributes to the homeostatic maintenance of secretory pathway function [38]. Despite ZnT5 has been shown to be expressed at the insulin granule level, genetic studies indicate that it is unlikely that its deletion has a direct effect on pancreatic beta cells [39]. Moreover, it was shown recently that overexpression of the Golgi-expressed zinc transporter ZnT7 increased the total cellular insulin content in the beta cell line RIN5mF [40]. ZnT7 overexpression led to a high basal insulin secretion but did not influence either glucose-induced insulin secretion or intracellular zinc content suggesting that the two zinc transporters may play different roles in the regulation of insulin metabolism in the β -cell. Recently, another zinc transporter, ZnT3, whose expression is regulated by glucose and zinc ions,

has been shown to be expressed in beta cells. Silencing of ZnT3 led to decrease insulin synthesis and secretion, for all the glucose concentrations tested [41].

1.3. Zinc and Insulin Signaling

While zinc is crucial for insulin synthesis and secretion, zinc ions play also a role in the insulin signaling in peripheral target tissues. Indeed, the action on insulin target tissue was discovered in the 1980's, when Coulston and Dandona studied the effect of zinc chloride on the rate of lipogenesis by rat epididymal adipocytes [42]. They found that zinc had a potent stimulatory effect upon lipogenesis, and that this effect was independent of, and additive to that of insulin. *In vivo*, ZnCl₂ was found to improve the hyperglycaemia of streptozocin-induced diabetic rats. In these rats, the insulin-like effect of zinc was observed on both stimulation of lipogenesis and the oxidation of glucose [43]. In genetically obese (ob/ob) mice, the effects of zinc supplementation were studied on plasma glucose and insulin levels, as well as its *in vitro* effects of lipolysis in adipocytes. Plasma glucose levels were reduced after zinc supplementation, as in many other studies (see below). Concomitantly, plasma insulin levels were significantly decreased by zinc treatment. *In vitro* analysis revealed that lipogenesis in adipocytes was significantly increased by up to 70%, suggesting that reduction of hyperglycaemia by zinc supplementation may be related to its effect on the enhancement of insulin action [44]. Therefore, compounds of metal complexes with antidiabetic properties have been developed (for review see [45]). Since the 1980s, the mechanism of the insulin-mimetic action of zinc has been examined by several groups, with respect to glucose oxidation, lipolysis stimulation, glucose transport, glycogen synthesis, etc. Altogether, these studies demonstrated that both the incorporation of zinc into cells and its subsequent interactions with several biological systems were essential for developing the insulin-mimetic activity of zinc [45].

At the cellular level, zinc increases total protein phosphorylation of the insulin receptor beta subunit of both preadipocytes and adipocytes [46], though toxic or non physiological concentrations of zinc were used. However, Maret et al. demonstrated that the inhibition constants of recombinant proteins were in the nanomolar range [47]. Therefore, zinc inhibition of tyrosine phosphatases occurs in the physiological range of available, exchangeable zinc (for review see [48]). Studies of protein phosphorylation state and tyrosine phosphatase activity showed that increases in cellular zinc provoked an increase in total protein tyrosine phosphorylation and inhibition of cellular phosphatases (see chapter 6). More importantly, zinc inhibited Protein Tyrosine Phosphatase 1B (PTP1B) with a IC₅₀ as low as 17 nM [49], suggesting that a 'zinc signal' can regulate insulin signaling downstream of insulin receptor in target tissues. Previous studies have shown that, similar to calcium, an intracellular release of free zinc, i.e. a zinc wave is involved in intracellular signaling events [50]. Zinc is therefore a potent physiological regulator of insulin signal transduction, mainly through its inhibitory effect on PTP1B, the key phosphatase that dephosphorylates the insulin receptor [51].

In the presence of zinc chelators, phosphorylation of insulin receptors is decreased in rat glioma cells. Interestingly, alpha subunit of the human insulin receptor was found to consist of several zinc finger motifs [52]. Insulin receptor substrate-1 (IRS-1) plays an important role as a scaffolding protein downstream of the insulin receptor. Zinc increases the tyrosine phosphorylation of IRS-1 in the presence or absence of insulin in

skeletal muscle cells [53]. Furthermore, it is evident that zinc activates Akt in several cell lines [54]. Phosphoinositide 3'-kinase/Akt (PI3K/Akt, also referred to as protein kinase B, PKB) is a serine/threonine protein kinase that plays a key role in multiple cellular processes, including glucose metabolism (for review see [55]). Metal-induced activation of PI3K/Akt was prevented by phosphatiylinositol-3-kinase (PI3K) inhibitors, while exposure to zinc enhanced PI3K/Akt activity in cells. PTEN is a phosphatase and a negative regulator of PI3K/Akt activity and studies showed that zinc treatment could downregulate expression of PTEN, thus possibly enhancing PI3K/Akt phosphorylation and activity [56]. Furthermore, PI3K/Akt axis is an integral component of the insulin signaling pathway in leading to such effects as proliferation and translocation of glucose transporter 4 (GLUT4) to the plasma membrane in order to facilitate glucose uptake. Interestingly, in 3T3-L1 adipocytes, PI3K/Akt was activated by zinc independent of any effects on IRS-1 [46]. Studies show that certain PKC isoforms are also activated by insulin and, similarly, inhibitors to this downstream target of PI3K/Akt prevent zinc-induced glucose transport [46]. Zinc also imitates insulin by stimulating nuclear exclusion of FoxO1a in human hepatoma cells, suggesting that expression of FoxO target genes is downregulated by zinc [54]. FoxO transcription factors are inactivated by Akt-dependent phosphorylation, resulting in nuclear exclusion of phosphorylated FoxO proteins and an attenuated expression of FoxO target genes, such as those of the gluconeogenesis enzymes glucose-6-phosphatase and phosphoenolpyruvate carboxykinase (for review see [57]). Furthermore, zinc induces glycogen synthesis primarily by phosphorylation of glycogen synthase kinase-3, which regulates the activity of glycogen synthase [54]. Another recent study shows that in adipocytes, zinc-induced inhibition of lipolysis is prevented by pretreatment with cytochalasin B, an inhibitor of GLUT4 [58], suggesting that zinc induced glucose uptake into the adipocytes by regulating multiple sites in the adipocytes. Actin filament rearrangement is important for GLUT4 translocation to the plasma membrane and interestingly cytochalasin D, which inhibits actin polymerization was shown to inhibit zinc stimulated glucose transport similar to that of insulin [46].

Thus, zinc activates many of the key mediators of insulin-like effects and as such there is increasing interest in developing zinc containing compounds with insulinomimetic activity. Fourteen day treatment with daily injections of zinc (II) complexes in KK-Ay mice (T2D model) maintained their blood glucose at normal range and improved glucose tolerance [59]. Similarly, blood glucose was normalized by intraperitoneal and oral administration of zinc (II) complexes in T2D GK rats [60]. Further, these compounds were also suggested to increase glucose uptake into adipocytes by affecting insulin receptor, PI3K/Akt and GLUT4 and inhibiting free fatty acid release by impacting phosphodiesterase activity [58]. Interestingly, a novel zinc (II)-dithiocarbamate complex was recently shown to not only improve the hyperglycaemia in KK-Ay mice, but also reduce, insulin, leptin, adiponectin and triglyceride levels [61], thus adding promise towards development of new anti-diabetic drugs based on these compounds.

2. Zinc Metabolism and Diabetes Mellitus

2.1. Zinc Content at the Beta cell Level

Insulin-secreting pancreatic beta cells are exceptionally rich in zinc, especially in secretory vesicles where zinc-insulin crystallization occurs. Beta cells secrete insulin in response to glucose and other secretagogues [14, 62]. During hyperglycaemia, the number of granules in the beta cells decreases in accordance with insulin secretion. Degranulation of the cells can be observed experimentally after administration of hypoglycemic compounds, such as sulfonylureas [63]. Under these circumstances, the disappearance of insulin from the beta cells is accompanied by loss of their zinc. Indeed, in 1974 Lazaris et al showed that administration of euglucon-5 or tolbutamide in rats dose dependently led to hypoglycaemia after 24 h. Staining of isolated islets by silver-sulfide method showed that insulin secretion was accompanied by an almost total loss of zinc from the beta cells [64]. However, the silver-sulfide method, like dithizone staining [65] lacked specificity and sensitivity. Despite using different zinc probes for staining of intracellular zinc pools (e.g. Fura-2, TSQ Toluene sulfonamidoquinoline), it is only in the early 1990's that the first generation of fluorescent, zinc-specific, intracellular probes were developed (see chapter 9). These probes were designed to visualize and quantify intracellular zinc pools in living cells. One of the breakthroughs in zinc visualisation and measurements came from Zinquin and derivatives [66]. Using these zinc-specific fluorophores, in conjunction with fluorescence digital image analysis, Zalewski et al were able to reveal labile zinc in frozen sections of pancreas and in isolated islets [67]. More importantly, the authors showed that exposure of islet or insulinoma cells to a high concentration of glucose or other secretagogue decreased the intracellular content of labile zinc in living cells. Therefore, at the cellular level, elevated glucose concentrations lead to dramatic loss in beta cell zinc content, along with insulin. Indeed, since zinc is crucial for insulin biosynthesis and storage (see above), and as zinc depletion can induce cell death ([68], and see chapter 5) an adequate supply in zinc is crucial for beta cells especially in hyperglycaemic conditions. It is likely that in conditions of insulin resistance, or in T2D, the increased insulin response participates in zinc loss from the beta cell. Pertinent to zinc content and insulin secretion, Quarterman et al. demonstrated fifty years ago that zinc-deprived rats displayed an impaired insulin secretion response to glucose stimulation [69]. It has also been demonstrated that in zinc-deficient states, there is a clear decreased islet cell insulin content, thus linking zinc status to insulin content in pancreatic beta cells.

2.2. Body Zinc Homeostasis

If beta cells tend to be depleted in zinc ions upon insulin secretion, a hallmark of diabetes is hypozincemia [70]. The decrease in total body zinc may result from either hyperzincuria, decreased intestinal absorption, and/or increased urinary excretion - principally due to zinc loss from beta cells. Indeed, when serum zinc levels were measured in a diabetic population (20 age- and sex-matched healthy controls and 30 diabetes mellitus patients), they were found to be significantly ($p < 0.001$) reduced as compared to healthy controls [71]. This study revealed that hypozincemia in diabetes mellitus may be due to altered zinc metabolism. A second study also demonstrated a clear hypozincemia on 20 patients with Type I diabetes. The authors showed that the zinc serum concentration was 56.6 µg/dL in diabetic patients, significantly lower than

the levels measured in matched controls (zinc serum concentrations in healthy controls are typically in the 70–110 µg/dL range [72]). Concomitantly, these patients displayed hyperzincuria, possibly explaining the observed hypozincemia [73]. Furthermore, the zinc deficiency occurring during diabetes might contribute to diabetic complications. Indeed, zinc deficiency might impair insulin biosynthesis and storage (see above), but will also impair immune function, mainly by interacting with pro-inflammatory cytokine production (see chapter 10). Zinc deficiency will also promote oxidative stress, since zinc will both protect sulphhydryl groups against oxidation and inhibit the production of reactive oxygens by transition metals [74]. It is of importance to note that oxidative stress generated by reactive oxygen species will significantly contribute to the pathogenesis of diabetes [75, 76].

2.3. Zinc Supplementation as an Oral Therapy

A potential role for oral zinc supplementation in therapy originates from the above studies on zinc content in diabetic patients. However, supplementation studies are few and most of them lack reproducibility, mainly because the dosage and the zinc species are different. Moreover, these studies are likely to be complicated (stratified) by polymorphisms, notably in the SLC30A8 gene [8]. High dose zinc supplementation of T2D patients e.g. 200mg X3/day during 7 weeks, significantly increased fasting plasma glucose, while bringing zinc and copper levels back to normal [77]. The authors concluded that zinc supplementation in T2D patients might aggravate glucose intolerance. The effects of zinc supplementation have also been studied in patients with T1D [78]. Here, again, despite daily dosages were based on Dietary Recommended Intakes, no positive effect on metabolic parameters were observed; an increase in HbA1C, a long term marker of blood glucose levels, was found. Another study on Type 1 diabetic patients reported a ‘disconcerting’ increase in metabolic parameters, e.g. HbA1C, after zinc supplementation (50 mg Zn daily for 28 days) [79].

On the other hand, a number of studies report beneficial effect for zinc supplementation in both types of diabetes, including animal models. In a study on diabetic patients with type I diabetes mellitus, zinc supplementation corrected the zinc deficiency. Moreover, after 3 months of zinc gluconate treatment (30 mg daily), markers of oxidative stress were decreased and antioxidant enzyme activity was increased, suggesting a protective effect of zinc [80]. In patients with T2D mellitus too, a significant decrease in different oxidative stress markers suggested beneficial antioxidant effects of zinc supplementation [81]. The antidiabetic effect of zinc was also described in a recent report, in which the authors investigated the effects of zinc supplementation (20mg daily) on insulin resistance and components of the metabolic syndrome. After receiving zinc, the mean fasting plasma glucose (FPG), insulin and HOMA-IR (a method usually used to quantify insulin resistance) decreased significantly [82]. Therefore, zinc supplementation was considered as a useful and safe additional intervention treatment for improvement of cardiometabolic risk factors. A more recent study in Berardinelli-Seip syndrome patients suffering from diabetes revealed that glycated haemoglobin decreased significantly, while plasma leptin, C-peptide (a surrogate marker for insulin) and serum zinc levels increased after zinc supplementation [83].

The effects of zinc supplementation have also been studied in animal models of diabetes, such as streptozotocin-induced diabetic mice (a chemically induced pancreatic injury to produce diabetes). While zinc treatment (20 ppm in drinking water for two

weeks) did not affect body weight gain, body fat content, it tended to increase serum leptin concentrations and markedly ameliorated the hyperglycaemia of diabetic mice [84]. Zinc supplementation in streptozotocin-diabetic rats (100 mg zinc sulfate/ kg body weight, every day for 60 days) caused a body weight increase. Nevertheless, zinc supplementation caused, here again, a marked decrease in hyperglycaemia after 2 months (218.55 ± 6.70 vs 126.14 ± 0.55 for diabetic controls and diabetic+zinc supplementation, respectively) [85]. Moreover, more insulin-immunoreactive cells were observed in the pancreatic islets of the diabetic 'zinc sulfate' group than in the diabetic 'control' group, suggesting that zinc supplementation may prevent diabetes in experimental animals. High zinc intake was shown to significantly reduce the severity of T1D (based on hyperglycaemia, insulin level, and islet morphology) in alloxan- and streptozotocin-induced diabetic models. Zinc supplementation also inhibited NFkB activation, which might be a key cellular process that bridges oxidative stress and the death of β cells [86]. Zinc supplementation was also proved efficient against diabetic complications. Indeed, it has been shown that the prevention of diabetic cardiomyopathy by zinc supplementation is predominantly mediated by a zinc-dependent increase in cardiac metallothionein [87]. More recently, Cai and colleagues tested whether induction of renal MT synthesis by Zn supplementation protects the kidney from diabetes-induced damage. Using streptozotocin-induced diabetic rats, they demonstrated that zinc supplementation (5 mg/kg zinc sulfate) attenuated diabetes-induced renal oxidative damage, inflammation, and prevented the kidney from diabetes-induced increases in 24-h urinary proteins and pathological alterations [88].

2.4. Zinc Metabolism, Oxidative Stress and Beta Cell Protection

Hyperglycaemia and hyperlipidemia that ensue during insulin resistance lead to increased metabolism and mitochondrial oxidation in beta cells, which leads to elevated mitochondrial membrane potential and superoxide production, increasing exposure to reactive oxygen species (ROS) [5]. Beta cell is equipped with uncoupling protein 2 (UCP2) to dissipate this mitochondrial membrane potential, thereby reducing ROS production [89, 90]. Although studies suggest that this uncoupling of oxidative phosphorylation could in fact lead to reduced insulin secretion, how UCP2 may affect insulin secretion still remains highly debatable. Beta cell has limited defense against excess ROS production because the expression levels of ROS-detoxifying enzymes in the beta cell are particularly low in comparison with those in other cells [76, 91]. Importantly, ROS is suggested as a key contributor to beta cell failure during insulin resistance.

Zinc is an important co-factor to many of these antioxidant enzymes such as catalase, glutathione peroxidise and especially, Cu/Zn-superoxide dismutase [92]. Thus, in many cell systems zinc is a suppressor of apoptosis [74, 93, 94]. However, a few studies report that zinc can induce islet cell death during hyperglycaemia [93]. Typically, under physiologic conditions, damage caused by zinc is negligible. Alternatively, zinc deficiency increases apoptosis in number of organs and tissues, while zinc supplementation has an inhibitory effect on apoptosis [68, 95]. Therefore, while zinc depletion reduced cell viability in isolated islets, pretreatment with zinc to increase the pancreatic zinc content, was shown to reduce the effects of streptozotocin-induced diabetes in rodents [84, 86, 96]. It is not known whether zinc supplementation affects the same pools and molecular targets within the apoptosis pathway as does zinc depletion. However, it is presumed that both excess and deficiency of zinc could

disrupt mitochondrial function resulting in oxidative stress. Thus, zinc deficiency observed during diabetes may provide an explanation to the reduction in beta cell mass associated with the disease. Zinc had been observed to provide protection at several levels in many cell types. Zinc is a potent inhibitor of the endonuclease responsible for apoptotic DNA fragmentation [97] and also protects sulfhydryl groups by forming bonds with thiolate complexes. As such, it is thought to interact with the sulfhydryl group required for catalytic activity of caspase-3, thereby disrupting its function [95]. Zinc is also a stabilizer of microtubules, and microtubular disruption occurs during zinc deficiency [98]. Zinc further functions to maintain adequate levels of metallothioneins (MT) [99]. Interestingly, polymorphisms in the zinc-buffering MTs have been recently linked to T2D [100, 101] (see below), suggesting that appropriate zinc metabolism is a mechanism relevant to the control of blood glucose. MTs demonstrate strong antioxidant properties [99]. Thus, studies suggest that zinc-metallothionein complexes provide cytoprotection against free radicals and oxidative stress in both alpha and beta cells [102, 103]. Interestingly, studies suggest that pro-inflammatory cytokines, a key mediator of beta cell death during diabetes, could dictate beta cell zinc levels [104, 105].

Another concept that has recently emerged is the possible relationship between islet amyloid and zinc. Islet amyloid deposition is a common occurrence in T2D islets. Zinc in connection with amyloid deposition in the brain has been studied at length. Interestingly, a recent study shows that zinc inhibits amyloid fibrillogenesis by increasing lag-time of fiber formation and decreasing the rate of addition of islet amyloid peptide to existing fibers [106, 107], thus possibly adding another dimension to the role of zinc in beta cell protection.

3. Zinc and Type I Diabetes

As discussed above, T1D is the most severe type of diabetes, mostly occurring during childhood though it may also be diagnosed in adults. It results from autoimmune attack on pancreatic beta cells, with subsequent destruction of these cells (for review see [108]). Therefore, hyperglycaemia occurs because the body is not able to produce insulin anymore, leading to life-long dependency on daily insulin injections. T1D diabetes is strongly genetically linked to genetics, and associated with HLA (Human Leukocyte Antigens) on chromosome 6 [109]. Given the strong association between T1D susceptibility and the HLA class II locus, investigators have long been focused on CD4(+) T cells, however recent studies point out a critical role of CD8(+) T cells in the pathogenesis of T1D [110]. Cells can be damaged by cytokine-induced free radical burst and/or appearance of autoantibodies against beta cell intracellular epitopes, both resulting in beta cell death [111].

Most of the components of the immune system, including beta cell autoantigens, macrophages, dendritic cells, B lymphocytes, and T cells, plays a central role in the pathogenesis of Type 1 autoimmune diabetes. Indeed, zinc ions play a key role in immune system function, and are therefore a key component of immune system-mediated beta cell destruction. The role of zinc in the immune system is comprehensively described in chapter 10 and will not be the focus of this chapter. Additionally, the natural history of islet autoimmunity and pathological mechanisms of T1D have been recently and extensively reviewed elsewhere [112, 113].

The most important markers for beta cell autoimmunity are circulating autoantibodies against insulin, glutamic acid decarboxylase (GAD65), and islet cell antigen-2 (IA-2, a tyrosine phosphatase-like protein), the number and levels of which are routinely used as predictive markers for the underlying autoimmunity that may precede T1D diagnosis in at-risk patients. Islet cell autoantibody assays, in particular the quantitative radioligand binding assays for GAD65 and IA-2autoantibodies, have also been important for diabetes classification [114]. Moreover, the combined biochemical measurement of auto antibodies to insulin, GAD65 and IA2 can identify 80% or more of patients at disease onset or at risk of developing disease [115]. Nevertheless, determination of sera circulating antibodies failed to determine individual risk in a general population; the search for new molecular targets is an important goal for clinicians, who need development of further markers of B and T cell autoimmunity in diabetes.

In 2007, a novel advance was made by John Hutton, George Eisenbarth and colleagues, who identified a novel marker based on a multidimensional analysis of microarray data, ZnT8. The authors identified anti-ZnT8 auto antibodies as early as two years of age, with increasing levels and prevalence persisting to disease onset [116]. Moreover, ZnT8 autoantibodies were persistent in the prediabetic phase and proved a useful independent marker of autoimmunity either alone in antibody-negative subjects or in conjunction with insulin, GAD65 or IA2 antibodies. Importantly, when combined, measurement of ZnT8, GAD65, IA2, and insulin antibodies raised autoimmunity detection rates to 98% at disease onset. This level approaches that needed to detect prediabetes in a general pediatric population. Indeed, it has become clear that autoantibodies to ZnT8 are detected in the majority of T1D patients prior to and at clinical diagnosis. There is now a consensus amongst diabetologists that the use of ZnT8 autoantibodies presents a useful marker for T1D, especially in younger patients at disease diagnosis [117]. Further, detection of ZnT8 autoantibodies helped to improve the prediction of a future insulin insufficiency in adult-onset autoimmune diabetes [118]. In a recent study in China, ZnT8 autoantibodies were recognized as independent marker for T1D Moreover, it has been suggested that presence of ZnT8 autoantibodies may be associated with different clinical phenotypes of T1D than GADA or IA-2A [119]. The determination of the four biochemically characterized islet autoantibodies [insulin, GAD65, IA2 and ZnT8] can now predict the development of T1D; prevention of T1D in animal models has also been achieved [120]. We can also prevent type 1A diabetes in animal models and the final goal is the prevention of type 1A diabetes in man

ZnT8 is an islet-cell-specific zinc transporter responsible for the uptake of zinc by insulin granule and participates in the regulation of insulin secretion ([30, 32]and see below). As such, it shares some specificities with other beta cell autoantigens, e.g. to be relatively specific to pancreatic beta cells and to be associated with elements of the regulated pathway of insulin secretion [121]. Assays for ZnT8 autoantibodies were developed using immunoprecipitation. Epitope mapping in T1D patients revealed that up to 70% of individuals had antibodies reactive to the carboxy terminal loop of ZnT8 [116]. The use of ZnT8 autoantibodies as an independent marker for autoimmune diabetes has been discussed, and the autoantibodies are usually preceded by GAD65Ab and IAA in the prediabetic period [122]. Further, it has been reported that the autoimmune response to ZnT8 is focused on a few key epitopes. Two of the main epitopes are defined by a polymorphic amino acid at position 325 (rs13266634), which is a key determinant of two of the three major conformational epitopes in the protein

[123], the third major epitope was not affected by aminoacid at position 325. Using sera from type 1 diabetic patients, authors showed that the autoantibody responses to the ZnT8 Arginine- and Tryptophane-restricted isoepitopes segregated with the alleles encoding the respective variant amino acids [123]. These results strongly suggested that ZnT8 autoantibody specificity is determined by a common SNP, rs 13266634.

Interestingly, and intriguingly, the same Single Nucleotide Polymorphism (SNP) at position 325 (rs 13266634) in SLC30A8 gene has also been linked to T2D by genome wide association studies [8], the importance of ZnT8 for beta cell function and its role in T2D are discussed below.

4. Zinc and Type II Diabetes

As discussed above, a relationship between zinc, the pancreas and diabetes was first suggested seventy years ago when a study showed that there is a 50 percent reduction in pancreatic zinc concentration in diabetic cadavers compared to non-diabetic cadavers [12]. Thirty years later, rodents fed a zinc deficient diet was shown to have reduced glucose stimulated insulin secretion and islet insulin content [69, 124]. Two years later, reduced beta cell granulation was also associated with zinc deficiency [36]. Interestingly, there is a reduction in plasma zinc concentration, high zincuria, and severe and ubiquitous cellular depletion of zinc in diabetic patients compared to healthy individuals [125], while zinc supplementation has positive effects on glucose handling in humans [126-129]. Reduced pancreatic zinc content is evident in several genetic mouse models of T2D [130-132]. Dietary zinc supplementation in db/db mice (mutation in the leptin receptor) was shown to normalize pancreatic zinc levels and attenuate hyperglycaemia and hyperinsulinemia, while a zinc-deficient diet had exacerbated fasting hyperglycaemia associated with reduced circulating insulin, suggesting a role of zinc in pancreatic function [132]. Similarly, zinc supplementation in ob/ob mice (mutation in the leptin gene) was also shown to elevate islet insulin content and attenuate fasting hyperglycaemia, hyperinsulinemia and the abnormally high insulin secretory response to glucose in isolated pancreatic islets [130].

Numerous studies have suggested that zinc associated MTs may contribute to diabetic complications associated with oxidative stress (see above). MTs are found at high levels in the pancreas and are induced upon streptozotocin (STZ) or cytokine treatment [133]. Interestingly, MT-null mice display reduced basal and glucose stimulated insulin secretion and became significantly obese by 6 weeks of age. Thus, it is not surprising that polymorphisms in MT are associated with T2D. One polymorphism in MT2A (rs1610216) was associated with T2D, lower circulating zinc and elevated glucose [101, 134]. Another SNP in MT1A (rs11640851), demonstrated in an Italian population was associated with T2D, higher glycaemia and MT levels and reduced intracellular zinc [100]. Another SNP in MT1A (rs8052394) associated with diabetes had reduced SOD levels [101]. Other polymorphisms in MTs have been associated with obesity, hyperlipidemia and elevated triglycerides in T2D patients [101, 134]. Interestingly, levels of MT1 and MT2 in the plasma and skeletal muscle of T2D patients were found to be significantly lower, while oxidative stress markers (malondialdehyde, 8-oxoguanine, and nitrotyrosine) were higher than those of controls. Moreover, a robust increase of MT-I+II in response to training has been observed in controls, while MT-I+II levels remained essentially unchanged in T2D, suggesting that the exercise-induced increase in antioxidant defense is impaired in T2D [135].

Recently the association between zinc and diabetes was further reiterated due to several genome wide association studies that suggested a zinc transporter in pancreatic beta cells as a risk locus for T2D [7-9, 136]. This referred to a non-synonymous SNP (rs13266634, R325W) in SLC30A8, which encodes ZnT8. The risk allele (R-variant) has an estimated prevalence of 55% in Asians, 75% in Europeans and 95% in Africans with association with T2D been mostly replicated in subjects of European and East Asian descent [137]. Interestingly, the major allele (also the risk allele) of SLC30A8 is associated with increased fasting plasma glucose, reduced glucose stimulated insulin secretion and impaired conversion of proinsulin to insulin, but not with insulin resistance [136-138]. Importantly, even individuals with one T2D parent homozygous for the major risk allele displayed a 19 percent reduction in first-phase insulin release during an intravenous glucose challenge, but no differences in second-phase insulin release or insulin sensitivity were apparent [139]. However, a report thereafter also described an association between SLC30A8 SNP rs13266634 and insulin sensitivity [140]. Interestingly, no relationship with SLC30A8 polymorphisms and indexes of beta cell function was found in several other studies [141, 142].

In vitro studies in pancreatic cell lines suggest that the R variant is a less efficient zinc transporter [33]. Interestingly, overexpression of ZnT8 in mouse pancreatic beta cells increased total cellular zinc content and glucose stimulated insulin secretion leading the authors to hypothesize that ZnT8 impacts zinc transport into insulin vesicles [32]. In agreement, two studies thereafter showed that ZnT8 down-regulation in beta cell lines reduced cellular zinc content and insulin secretion in response to glucose [104, 143]. One of them also showed reduced cellular insulin content and fewer dense core granules [143]. Souza et al. also suggests that overexpression of ZnT8 in an alpha cell line inhibits glucagon secretion by 50 percent, while siRNA knockdown of the protein stimulates glucagon secretion by 70 percent [144]. Thus ZnT8 may also directly regulate glucagon in addition to insulin secretion. Studies looking at the impact of ZnT8 on pancreatic beta cell survival have been variable. While overexpression of ZnT8 had a protective effect on zinc depletion-induced cell death [32], it had rendered cells more susceptible to IL1 β induced apoptosis and was not protective with respect to cytokine exposure [105].

Further insight into the physiological role of ZnT8 with respect to beta cell function and maintenance of glucose homeostasis was gained by characterization of mice with ZnT8 deletion. These mice were hyperglycemic and glucose intolerant, had abnormal insulin granule morphology, reduced zinc accumulation in islets and glucose stimulated insulin release [33]. Thus these studies suggested that lack of ZnT8 has an impact on insulin crystallization leading to either abnormal or complete lack of crystallization, possibly due to the reduced transport of zinc into these granules. Although deficiencies in islet zinc accumulation was common in all colonies of ZnT8 knockout mice, subtle phenotypic differences existed between groups. Glucose intolerance was not apparent in one group which studied much older mice [35] and was less marked in another which maintained the colony on a background enriched in SV129 strain [34]. In the latter group, glucose intolerance was apparent only after the induction of a metabolic stress (high fat diet). Thus, the differences may have been attributed to such environmental differences as age, genetic background (number of backcrosses into C57BL6 background) and the composition of the diet. We have in fact observed that mice from different backgrounds may express ZnT8 at different levels [29, 33, 103, 145]. Surprisingly, impaired in vitro insulin secretion was apparent only in one of the studies, suggesting that the conditions used in vitro failed fully to mimic

the stimulation of secretion as occurs *in vivo*. This not so striking change in insulin secretion raises the question, why does physiology favour insulin-zinc crystallization?. One possibility is that zinc may affect the potency or bioactivity of insulin and the rate of its degradation in plasma. In both hagfish and guinea pig, who do not form zinc-insulin crystals, bioactivity and potency of insulin is significantly lower than other mammalian insulin [22, 146]. Thus in the guinea pig, there is a compensatory increase in insulin secretion [146]. Similarly it was previously shown that there is less insulin-like activity in serum of zinc deficient rats [147]. On the other hand, beta cell selective deletion of ZnT8 [29] led to similar morphological and metabolic changes as observed above, but in this strain insulin secretion defects were also apparent in isolated islets. These observations may suggest that ZnT8, also present in alpha cells [33], plays a role in the “capture” of zinc released from beta cells along with insulin (it is of note that roles for zinc in the control of glucagon secretion in the rat [148], have not been observed in other mammalian species including mouse [149] and human [150]. Thus, deletion of ZnT8 in beta cells, when uncompensated by loss from alpha cells, may lead to more drastic decreases in intracellular zinc. Surprisingly though, we thus far have not observed any changes in plasma glucagon levels or glucose homeostasis in mice lacking ZnT8 specifically in alpha cells [29]. Correspondingly, beta cell-specific ZnT8 knockout mice revealed decreases in the expression of beta cell enriched genes involved in insulin gene transcription (MafA, Pdx1) as well as insulin processing (prohormone convertases PC1, PC2).

One major limitation of these studies is that mouse ZnT8 has a glutamate at the 325 position unlike in human. Thus, physiological role of ZnT8 in mouse may be different compared to a human and thus, eliminating ZnT8 in the mouse may not necessarily reflect the full impact of ZnT8 on human physiology. Therefore, given the imperfect nature of mice (lifespan <3 years, mass <30g) as models of human disease, the appearance of an aberrant glucose homeostasis is consistent with the increased risk of T2D in carriers of a hypomorphic SLC30A8 alleles. The stronger phenotype in humans may be attributed to the convergence of this defect with both environmental and epigenetic factors. Thus it seems reasonable to hypothesize that impaired insulin processing, and possibly the formation of amyloidogenic particles, underlie the increased risk of diabetes in carriers of risk SNPs in the SLC30A8 gene [151].

These genetic studies in humans, dietary studies in rodents and knockout mouse models suggest that zinc plays a primary role in the pathogenesis of diabetes and is indeed an essential micronutrient in maintaining proper glucose homeostasis. Therefore, considering the positive outcomes of zinc supplementation, especially with insulin [70] mimetic abilities of zinc, this micronutrient may be a new candidate to be utilized in diabetes therapy and prevention.

5. Conclusion and Perspectives

Diabetes mellitus, one of the most common chronic diseases, is a multifactorial disease with various contributing factors. Regardless of the etiopathology of the disease, it is obvious that zinc plays a crucial role in the maintenance of blood glucose level. As discussed above, zinc is an essential component of the insulin biosynthesis machinery. It is required for almost all processes, from insulin gene transcription to insulin-zinc crystallization in secretory granules. In T1D, zinc ions play a key role in immune system function, and are therefore a key component of immune system-mediated beta

cell destruction (see Chapter 10). Identification of ZnT8 autoantibodies against a zinc transporter, ZnT8, helped to improve prediction and diagnostic of T1D. Moreover, using GAD 65 as a target, clinical trials are underway aimed to prevent beta cell destruction, and antigen-based immunotherapy provides an approach to selectively tolerize pathogenic beta cell-specific T cells. It might be possible that in the future this zinc transporter will participate in the prevention of the disease. Regarding T2D, the longstanding association between zinc and diabetes was further reiterated at the genetic level by identifications of polymorphisms in an islet-specific zinc transporter, ZnT8, as in the zinc-buffering metallothionein family of protein in T2D patients. Clinical studies have linked the polymorphism in ZnT8 to insulin secretion and/or beta cell function. Screening of at-risk alleles in the general population, or in specific subgroups, might allow identification of at risk patients and implementation of prevention strategies for T2D. Zinc supplementation has also been shown to have beneficial effects on blood glucose levels in both types of diabetes, in animal models and humans. Though supplementation is not specific for the targeted organ/cells, and while it remains unclear whether the effects of supplementation target insulin synthesis/secretion, insulinomimetic effects or beta cell protection, or possibly all three, zinc emerge as an efficient tool for diabetes prevention and therapy. Future strategies for specifically targeting zinc ions to desired tissue/cells, either using small molecules targeting zinc transporters or technologies that allow targeted delivery of zinc in targeted tissues may improve the potency of zinc in the prevention and treatment of diabetes.

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26. Zinc and Skin

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Abstract. Zinc has been empirically used to treat skin afflictions for thousands of years. However, even today, the mechanisms by which zinc influences physiological and pathological skin conditions are not entirely known. In this chapter, the association of zinc deficiency and skin symptoms will be discussed, as well as the role of zinc concerning wound healing taking the underlying mechanisms into account. Furthermore, an overview of possible therapeutic applications of zinc regarding selected skin disorders will be given.

Keywords. Zinc, Skin, Wound healing, Skin disorders

Introduction

Zinc has been empirically used as therapeutic agent supporting wound healing for more than 3000 years – the ancient Egyptians used calamine for topical application of zinc – but it was not until the 1930s that systematic analyses concerning the association between zinc and skin pathologies were initiated [1,2]. In 1933, zinc deficiency could be related to skin symptoms when Todd et al. showed that young rats receiving a zinc deficient diet suffered from hair loss affecting especially the ventral side of the body [3]. Hair loss can be regarded as skin symptom, since hair, as well as nails, represent appendages of the skin. Particularly during the last 50-60 years, studies concerning the impact of zinc on wound healing and its therapeutic options have been conducted [4].

The importance of zinc for human skin is further emphasized by the fact that the zinc level in skin is among the highest in the human body, making up approximately 6% of the total body zinc following skeletal muscle and bone [5,6]. This zinc is present mainly in the epidermis, since the zinc content of the underlying dermis was shown to be markedly lower [6,7]. Regarding the essential role zinc plays in gene expression, cell proliferation and signal transduction [8-10], it is not surprising that such a great amount of zinc is present in the highly proliferating epidermis [11].

This chapter intends to give insight into the association of zinc with skin concerning zinc deficiency, wound healing and skin disorders as well as into potential therapeutic applications of zinc today.

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1. Skin Symptoms Related to Zinc Deficiency

Zinc deficiency can be caused by numerous conditions. One genetically determined, rare disorder resulting in zinc deficiency is acrodermatitis enteropathica, which is inherited in an autosomal recessive way. The affected gene is that of ZIP4 (Zrt-, Irt-like protein 4), a zinc transporter responsible for zinc uptake in the intestine [12-14]. Typical symptoms of acrodermatitis enteropathica comprise gastrointestinal disturbances such as diarrhea, malabsorption and steatorrhea; neuropsychiatric symptoms such as irritability and tremor; growth retardation; disturbed immune function; ophthalmic affections such as blepharitis, conjunctivitis and photophobia; and skin manifestations [13,15,16]. These affections of the skin include generalized alopecia, angular stomatitis, glossitis, perioral as well as acral rash, scale, erythema, erosions, impaired wound healing, paronychia and vesiculo-bullous eruptions. The skin of the extremities as well as of the oral, genital and anal region seems to be predominantly affected. Additionally, infections with the fungus *Candida albicans* occur quite frequently. All of the enumerated symptoms are remarkably regressive under zinc supplementation, which is the life-saving treatment of acrodermatitis enteropathica [13,15,17].

Other causes of zinc deficiency may be eating disorders such as anorexia nervosa [13,18], malabsorption syndromes, lack of dietary zinc intake which is especially frequent in developing countries and among elderly people, cirrhosis of the liver, chronic renal disease, alcohol abuse, total parenteral nutrition, extensive skin burns and others [15-17,19-21]. The symptoms resulting from those acquired states of zinc deficiency are similar to the symptoms of acrodermatitis enteropathica described above. Those manifestations of acquired zinc deficiency can be treated and prevented by zinc supplementation [15,16], but the treatment of the underlying condition causing this zinc deficiency also has to be taken seriously.

2. Zinc and Wound Healing

The importance of zinc regarding the wound healing process can be concluded from study results demonstrating that zinc deficiency in rats leads to reduced zinc content of the skin and results in delayed wound healing as well as in a reduction of tensile strength compared to control animals fed a normal diet containing sufficient zinc [22-24].

Consistently, systemic zinc supplementation in form of oral zinc sulphate has been shown to accelerate wound healing in patients after excision of a pilonidal sinus [2]. In a small study analyzing the effect of oral zinc sulphate on the healing process of burns, a tendency towards reduction of healing time under zinc supplementation was observed [19].

However, several studies conducted with rats are not entirely in accordance with these findings. Those rats, which were not zinc deficient and were fed a regular zinc-containing diet, did not show any improvement of wound healing or tensile strength when zinc was supplemented systemically [24-26]. Thus, these results suggest that zinc supplementation might only have a beneficial effect on wound healing if there is an underlying zinc deficiency [15,27]. This could in fact be the case in the study examining burns described above, since patients suffering from skin burns usually develop a zinc deficiency due to losses in exudates [15,19].

This thesis is further supported by several studies examining the effect of zinc on venous leg ulcers. Greaves and Skillen observed a significantly lower plasma zinc level in patients suffering from venous leg ulceration resistant to treatment. Supplementation with 660mg zinc sulphate increased plasma zinc and induced wound healing in this uncontrolled study, but the rate of healing was unrelated to plasma zinc concentration. Except for nausea, no further toxic adverse effects were documented [28]. In a placebo controlled trial, zinc sulphate has also been shown to enhance wound healing in patients suffering from venous leg ulcers [29]. Although plasma zinc was not measured in this study, the patients showed an average age of 62.05 years in the control group and 60.34 years in the treatment group indicating that many elderly people were included among which mild zinc deficiency is rather frequent [21,29]. Another controlled study only indicated a better healing rate of leg ulcers under zinc supplementation without statistical significance. Plasma-zinc was not measured, yet the average age of patients again indicated an elderly study population. Adverse effects were not reported [30].

Two studies analyzing zinc sulphate versus placebo in chronic venous leg ulceration could not demonstrate an association between low serum zinc and delayed wound healing, which might be due to the variety of other factors that are crucial to a proper healing process. Furthermore, oral zinc sulphate supplementation did not improve wound healing in contrast to the studies just described [31-33]. But since the mean serum zinc of those patients was well within the normal range, an underlying zinc deficiency is unlikely and so these findings do not refute the theory discussed above, namely that zinc supplementation is especially effective in patients displaying a certain degree of zinc deficiency [31,32].

Regarding topical application of zinc for the purpose of improving wound healing, which was thoroughly examined using animals, results of studies are contradictory. On the one hand, beneficial effects of zinc on wound healing and on healing of burns in terms of increased re-epithelialization, reduction of bacterial growth, less inflammation, dissolution and removal of necrotic tissue were demonstrated [34-38].

On the other hand, there are a few studies displaying a lack of effect or even a negative influence of topically applied zinc [35,39,40]. Treatment of wounds with zinc oxide powder in rats did not result in any better outcome compared to untreated animals [40]. Zinc oxide in a hydrocolloid dressing did not have any effect on epithelialization of wounds in domestic pigs when the concentration of zinc was 2-6% and it even inhibited epithelialization when the zinc oxide concentration was below 1% [35]. Furthermore, an adhesive zinc tape was shown to influence the wound healing process by reducing wound contraction in comparison to treatment with a sterile gauze sponge [39]. This does not necessarily have to be caused by the zinc present in the adhesive zinc tape, but could also be due to the way this adhesive zinc tape was attached to the wound. It was observed that a tape that was fixed to the surroundings of the wound inhibited wound contraction in contrast to a tape that only covered the wounded area without contact to the surrounding skin [41].

These inconsistencies might be due to the mode of zinc delivery, as zinc oxide was shown to increase epithelialization of wounds whereas zinc sulphate rather inhibited epithelialization [34]. Furthermore, the type of the zinc-containing vehicle and its attachment to the wound could account for some of the differences, discrepancies in the wound healing process between species have to be taken into account, as well as the dosage of zinc applied [34].

A relatively consistent finding resulting from those studies examining animal wounds treated with topical zinc was the increase of serum zinc indicating absorption of zinc from its application site [34,40-42]. This absorbed zinc is suggested to promote wound healing, which is concluded from the following observation: A wound located at the dorsolateral side of a rat's back covered with a plastic foil or plastic tape showed a faster wound healing when a second wound at the other side of the back was covered with an adhesive zinc tape instead of plastic foil/tape. Wound healing underneath the zinc tape was slower than underneath the plastic foil/tape, so it was concluded that a high local zinc concentration might inhibit the formation of granulation tissue necessary for wound healing [41,42]. Indeed, zinc was found to be increased in granulation tissue of wounds treated with a zinc tape compared to wounds treated with a sterile gauze sponge [39]. However, local zinc application using a zinc tape did not return wound healing to normal in rats suffering from zinc deficiency suggesting that in this case systemically administered zinc is necessary to overcome the zinc deficit [39].

Studies including patients suffering from leg ulcers of different origin supported the positive effect of zinc on wound healing since, for example, leg ulcers treated with a zinc oxide dressing in comparison to a placebo dressing, showed faster wound healing and a lower rate of infections without relevant adverse reactions [43]. In a further study, leg ulcers showed a clear tendency towards faster healing and increased epithelialization when treated with a zinc-saline wet dressing instead of a normal-saline wet dressing [44].

Although differences in study design complicate comparison of different studies and despite a few studies not showing any effect or even displaying a negative influence of zinc on healing, the positive effect of zinc on wound healing under certain conditions, especially in patients displaying zinc deficiency, cannot be denied. In addition, the observation that zinc was found to be increased in wounded skin compared to healthy skin in animals indicates the importance of zinc for the process of wound healing [35,45,46].

The mechanism of wound healing and the role zinc plays in this process will be further discussed in the following sections.

2.1. Mechanism of Wound Healing

The process of wound healing can be subdivided into four phases in order to better understand the cascade of events occurring at a cellular level during healing of acute wounds [33].

The "Coagulation and Haemostasis Phase" occurs as immediate response to the injury and, as the name indicates, it comes to platelet activation and aggregation, subsequent coagulation leading to a fibrin clot and finally to haemostasis in the wound region in order to prevent exsanguination. The granules of platelets contain growth factors such as TGF (transforming growth factor)- β or PDGF (platelet derived growth factor) that are released and initiate attraction and activation of inflammatory cells such as neutrophils and macrophages, as well as of structurally relevant cells such as endothelial cells and fibroblasts. Other substances stored in these granules, serotonin for instance, cause vasodilatation and fluid extravasation leading to oedema. Furthermore, as a consequence of injury to cell membranes, eicosanoids are released, which play an important role in the following inflammatory response [27,33,47,48].

The "Inflammatory Phase" has the purpose of defending the wound, which is a defect of the protective skin barrier, against invading pathogens. Starting parallel to late

coagulation, neutrophils invade the wound within 24-36 hours after injury in order to fight invading pathogenic agents by phagocytosis and release of reactive oxygen species. About 48-72 hours after injury, macrophages are discovered in the wound continuing phagocytotic processes and stimulating regeneration by activating keratinocytes, endothelial cells and fibroblasts mediated through secretion of growth factors, chemotactic factors and other mediators. Thus, macrophages seem to mediate the transition between inflammation and reparation processes. Lymphocytes appear in the wound region after 72 hours. Indispensable factors for the migration of immune cells are integrins, which are necessary for adhesion of cells to endothelial cells thus allowing diapedesis of leukocytes out of the blood vessels into the surrounding wounded tissue and for adhesion to the extracellular matrix [33,47].

The "Proliferative Phase", which begins approximately on day three after wounding and lasts for about 2 weeks, is characterized by migration of fibroblasts and synthesis of new extracellular matrix resulting in formation of granulation tissue. The migration of fibroblasts, again, is dependent on integrins, which mediate adhesion to the extracellular matrix. Collagens are synthesized by fibroblasts in order to stabilize the newly built tissue. Angiogenesis, the creation of new blood vessels, is promoted by a number of endothelial factors such as FGF (fibroblast growth factor) or VEGF (vascular endothelial growth factor) and is crucial to the formation of healthy granulation tissue, which serves as a "provisional wound matrix". Granulation tissue consists of proliferating fibroblasts and vascularized stroma, macrophages, fibrinogen, fibronectin, collagen and hyaluronic acid. In the course of the healing process, fibroblasts differentiate and become myofibroblasts thus leading to wound contraction resulting in reduction of the epithelial defect. From the edges of the wound, migration of epithelial cells starts and the skin barrier defect is covered by a new layer of epithelial cells. For this migratory process, integrins play a pivotal role [33,47,48].

The final phase is the "Remodeling Phase". It can last up to two years or even longer and it comprises the formation of new epithelium including a new base membrane and eventually formation of scar tissue. This process has to be tightly regulated since a balance has to be maintained between synthesis and degradation of tissue, especially collagen. MMPs (matrix metalloproteinases) are synthesized by macrophages and fibroblasts and they degrade collagen. Inhibitory factors, which increase with the progression of wound healing, control their action. Through the activity of MMPs, the initially irregular collagen matrix of the granulation tissue, which mostly consists of type III collagen, becomes more organized and type III collagen is replaced by type I collagen. Gradually, the number of fibroblasts/myofibroblasts and macrophages in the wound is reduced by apoptosis and the blood supply decreases. The final scar is characterized by a diminished number of cells and blood vessels compared to normal tissue and a high tensile strength mediated by the predominating type I collagen [33,47,48].

2.2. Role of Zinc Concerning the Wound Healing Process

Zinc and zinc-containing proteins seem to be involved in almost every step of wound healing [11]. In order to analyze the action of zinc during the wound healing process, zinc concentration in skin during the course of healing has been examined in several studies.

In wounded rats, zinc was found to be increased in injured skin compared to normal skin especially during the first seven days after wounding. Then, the zinc

content measured in injured skin gradually decreased until reaching the zinc levels of normal skin on approximately day twelve [46].

Consistently, Iwata et al. showed an increase of zinc as well as of MT (metallothionein) and MT mRNA in wounded skin of mice from day three to day seven after injury [45]. MT is a zinc binding protein responsible for zinc homeostasis by tightly binding zinc, on the one hand, and releasing it depending on the redox state, on the other hand. It can be regarded as a reservoir of zinc (see chapter 4) [9,11,49]. As MT was mainly expressed in proliferating epidermal cells, it can be concluded that zinc is necessary for epidermal cell proliferation [45].

A similar pattern of changes in zinc concentration concerning wounded skin was described by Lansdown et al., who used a rat model for their studies [50]. They, too, observed an increase in MT up to the fifth day of wound healing. MT was located especially in epidermal cells at the wound margin and in regenerating tissue as well as in fibroblasts and macrophages of the upper dermis. These observations support the involvement of zinc in epidermal proliferation and indicate the importance of the metal concerning inflammatory processes [50].

Comparing the results of these studies to the time flow of wound healing, it becomes obvious that zinc concentration in injured skin is increased predominantly during the inflammatory and proliferative phase of the wound healing process supporting the proposed impact of zinc on inflammation and proliferation [50,33].

These findings are not surprising since zinc is known to be critical for the function of over 300 hundred enzymes including members of all enzyme classes thus being involved in inflammation and proliferation [10,51].

It is well-known that zinc influences immunological and inflammatory processes by modulating, for instance, signal transduction and cytokine secretion (see chapter 6) [8,52-54]. In a study examining cutaneous wound healing in mice, zinc deficiency led to delayed wound closure, to reduction of mRNA of the proinflammatory cytokines IL (interleukin)-1 β and TNF (tumor necrosis factor)- α and to decreased infiltration of neutrophils during the early "inflammatory phase" of wound healing. Zinc supplementation (500 μ g/g diet) exerted positive effects on the inflammatory response and on wound closure, but supplementation with 1000 μ g/g zinc negatively influenced inflammation and wound healing indicating that zinc supplementation only exerts positive effects when an adequate dose is applied [55]. This dose-dependency of zinc-effects on immunological processes has been described previously [51,54]. For further information concerning the involvement of zinc in immunobiology please refer to chapter 10.

Since zinc is required for the function of DNA-/RNA polymerases as well as for zinc finger motifs of transcription factors which modulate gene expression it is necessary for DNA/RNA-synthesis and cell division (see chapter 5) and thus the involvement of zinc in epidermal proliferation is not an unexpected observation, either [4,8,10,11].

Zinc-dependent transcription factors that have been linked to healing of skin wounds are, for instance, basonuclin, c-Krox and Egr-1 [56-60]. Basonuclin is a transcription factor consisting of six zinc finger motifs. It is predominantly expressed in basal keratinocytes of stratified squamous epithelia. It could be shown to be associated with the proliferative capacity of keratinocytes not yet terminally differentiated [57,58]. Another zinc finger-containing transcription factor found in skin is c-Krox. This factor seems to regulate transcription of fibronectin as well as collagen transcription by binding to the collagen I gene promoters. Consequently, this transcription factor

interacts with synthesis of extracellular matrix which is an important part of wound healing [56,59]. Egr-1 (early growth response-1), also called Krox 24, is a DNA-binding protein containing zinc fingers that is supposed to play a role in proper wound healing since Egr-1-null-mice showed reduced wound contraction, reduced collagen deposition, lower breaking strength of the wound and a lack of differentiation of fibroblasts into myofibroblasts. Consistently, overexpression of Egr-1 in mice resulted in increased deposition of collagen as well as in greater tensile strength [60].

The pivotal role of zinc in proliferation of epidermal keratinocytes is further underlined by a study investigating experimentally induced epidermal proliferation using MT-null mice. Whereas in normal control mice the intensity of MT staining increased in the proliferating epidermis, MT-null mice did not show any relevant staining of MT and a significantly lower epidermal thickness compared to control mice. In addition, consistent with the lack of MT, the skin of MT-null mice contained less zinc compared to normal mice [61]. Furthermore, ZnCl₂ up to 100µM was shown to stimulate proliferation of HaCaT cells, an immortalized human keratinocyte cell line. Correspondingly, the zinc chelator TPEN (NNN'N'-tetrakis(2-pyridyl-methyl)ethylene diamine) inhibited cell proliferation and this effect was abolished when TPEN was combined with zinc [62]. Finally, zinc sulphate and zinc histidine were found to enhance proliferation of normal human keratinocytes and to induce keratinocyte differentiation which occurs when keratinocytes move upwards from the basal proliferative cell layer to eventually form the superficial horny layer of the skin [63].

In addition, the involvement of zinc in wound healing can be related to two specific protein classes, integrins and MMPs, that are dependent of zinc and indispensable for the process of wound healing [33,47].

Integrins are heterodimeric transmembrane proteins consisting of an α- as well as a β-chain. They mediate cell-cell and cell-matrix interactions and are capable of initiating signaling pathways. In wound healing, they are crucial to migratory processes concerning leucocytes, endothelial cells, fibroblasts and keratinocytes [47]. Of special interest concerning cutaneous wound healing are the following integrins expressed on keratinocytes: α₂β₁, α₃β₁, α₆β₄ and α_vβ₅ [64]. Zinc was found to dose-dependently modulate the expression of these integrins, which are especially responsible for cellular mobility in the “proliferation phase” of wound healing. Low zinc doses (0.9µg/ml) led to an insignificant decrease of integrins (α₂, α₃, α₆, β₁) whereas a higher dose (1.8µg/ml) significantly increased integrin-expression (α₂, α₃, α₆, α_v) [64]. Consistent with these results, 1.8µg/ml zinc stimulated migration of keratinocytes and this migration was disturbed by addition of inhibitors of certain integrin subunits indicating that zinc exerts its impact on keratinocyte-migration by regulating integrin-expression [65].

MMPs are zinc dependent endopeptidases consisting of a highly conserved zinc binding site in the catalytic domain. They are crucial to a proper wound healing process. Their capability of degrading almost all components of extracellular matrix such as collagen, laminin, elastin and fibronectin underlines their importance for detachment and migration of cells as well as for tissue remodeling and re-epithelialization, which are processes present predominantly in the “remodeling phase” of wound healing [11,33,66,67]. MMPs can be synthesized by fibroblasts, macrophages, endothelial cells, neutrophils, eosinophils and mast cells in skin and they are released as response to stimuli such as cytokines, growth factors or changes of cell-cell contact [67]. Twenty-three human MMPs have been identified so far [68]. The MMP-family can be divided into collagenases, gelatinases, stromelysins, matrilysins, MT (membrane-type)-MMPs

and others based on their substrates and domain organization [69]. The most relevant MMPs for wound healing comprise collagenases (MMP-1, MMP-8, MMP-13), stromelysins (MMP-3, MMP-10), gelatinase A (MMP-2) and gelatinase B (MMP-9) [70]. The importance of zinc for proper function of MMPs could be demonstrated in a study using chelating agents in order to chelate zinc. This resulted in inhibited enzyme activities of the MMPs examined. Consequently, zinc seems to be necessary for the function of MMPs and thus for a regular process of remodeling during wound healing [71].

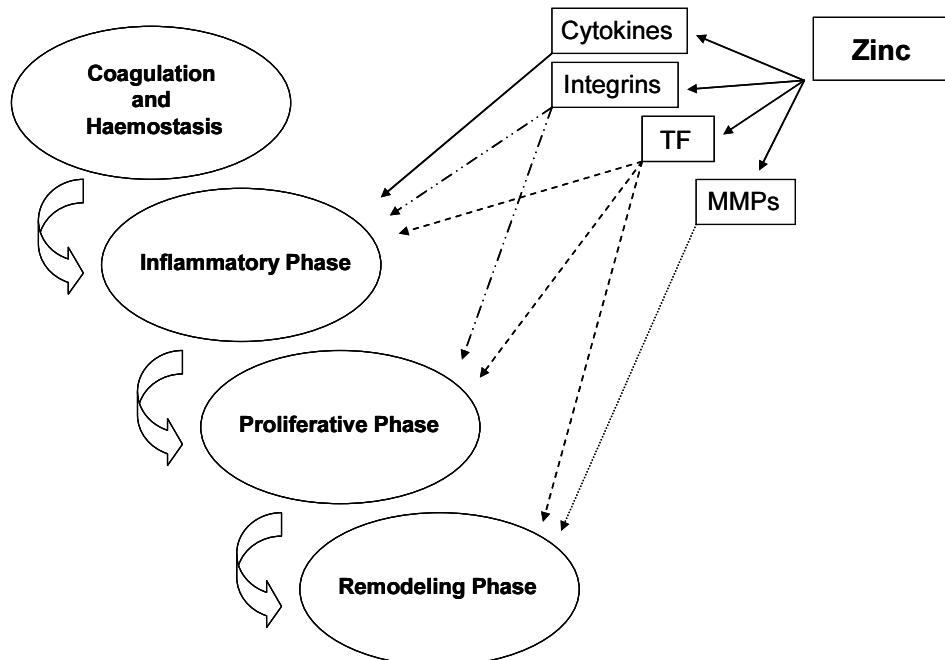


Figure 1. Overview of the role of zinc in the process of wound healing. Abbreviations: TF, transcription factor; MMPs, matrix metalloproteinases

In summary, zinc takes part in almost every phase of wound healing by modulating inflammatory processes occurring in the “inflammatory phase”, influencing gene transcription and integrin expression especially present in the “proliferative phase” and by being necessary for the function of MMPs, which are mainly active in the “remodeling phase” (figure 1).

3. Anti-bacterial Effects of Zinc

Anti-bacterial effects are desirable in treatment of skin alterations and disorders, especially taking into account that skin disorders, for example acne vulgaris, may be characterized by bacterial colonization [72]. Furthermore, an anti-bacterial agent would

be preferable for treatment of skin ulcers, burns and other conditions associated with a damaged skin barrier in order to prevent infections of the wounded skin [33].

The anti-bacterial effect of zinc was shown in *in vitro* experiments by Söderberg et al. who found that zinc oxide inhibits bacterial growth in disc diffusion tests, agar dilution tests and broth dilution tests. Most susceptible to zinc oxide were *Staphylococcus aureus* and *Staphylococcus epidermidis*, whereas streptococci and gram negative bacteria were clearly less affected by zinc oxide. In all cases high concentrations of zinc oxide were needed [73]. Additionally, excised wounds in rats showed a significantly lower number of bacteria when they were treated with an occlusive zinc dressing instead of an occlusive hydrocolloid dressing or sterile gauze sponge [74]. Determination of the minimum inhibitory concentrations of zinc for bacteria isolated from rat wounds and from wound infections as well as urinary tract infections in humans revealed that *Streptococcus* groups A, C, G are most susceptible to zinc followed by *Staphylococcus aureus* and *Streptococcus* group B, then *Escherichia coli* and finally *Pseudomonas aeruginosa* [74]. The differences concerning the susceptibility of streptococci to zinc might be due to differences in strains [73,74]. Consistent to the findings just described, analysis of skin biopsies taken from guinea pigs with third degree burns, which have been infected with *Pseudomonas aeruginosa*, indicated an anti-bacterial activity of zinc concerning *Pseudomonas aeruginosa* as well as *Staphylococcus aureus*, the latter being considered contaminating bacteria [37].

A possible mechanism by which zinc leads to inhibition of bacterial growth comprises the involvement in immune processes including signal transduction and cytokine release [11,52,54,55].

4. Zinc and Skin Disorders

Topical zinc has been used for centuries for treatment of various skin alterations, but the scientific background of the observed beneficial effects still has not been entirely elucidated [11].

Findings that support the usefulness of topically or systemically applied zinc in treatment of skin diseases comprise low serum zinc concentrations in patients with diverse skin conditions [75-79]. Children with atopic eczema displayed a significantly lower serum zinc concentration compared to healthy controls, but there was no evident correlation between serum zinc and severity of eczema [76]. Patients with severe acne vulgaris, which is characterized by more inflammatory lesions compared to low-grade acne vulgaris, also showed a decreased serum zinc level [75,80]. Additionally, significantly lower serum zinc concentrations were observed in patients suffering from chronic venous leg ulcers, recalcitrant viral warts, psoriasis vulgaris or ichthyosis in comparison to controls [77,79,81]. Psoriasis vulgaris is a chronic inflammatory skin disease that can result in many patterns of skin alteration, most commonly in red elevated plaques with scaling [82]. Ichthyoses are scaling disorders of diverse etiology characterized by abnormal desquamation and accumulation of scales [83]. Even neutrophil zinc concentration, which is supposed to better describe the tissue zinc status compared to plasma or serum zinc, was significantly reduced in patients with psoriasis compared to control patients and to patients with seborrhoeic dermatitis. The neutrophil zinc level in psoriatic patients did not depend on severity of psoriasis [78]. From these findings, it could be concluded that a certain zinc deficiency is present in patients with

those dermatologic conditions mentioned above and that systemic or topical application of zinc might have a beneficial therapeutic effect concerning skin alterations.

4.1. Systemic Application of Zinc

Systemically applied zinc has been examined regarding rosacea, acne vulgaris and recalcitrant viral warts [81,84].

Rosacea, a chronic inflammatory disease of the facial skin leading to flushing, papules, pustules or teleangiectasies, improved under zinc sulphate supplementation indicating a potential therapeutic effect concerning this skin disease. The effect might be due to modulation of inflammation or effects on oxidative status mediated by zinc [84,85].

Acne vulgaris is a common disorder showing colonization with *Propionibacterium acnes* (*P. acnes*) and an immune response of the body resulting in inflammatory lesions while also non-inflammatory comedones can be found [72]. Thus, anti-inflammatory effects are desirable and zinc as an anti-inflammatory and anti-bacterial agent was considered for therapy [72]. Recently, zinc was found to decrease TLR-2 (toll-like receptor-2) expression by keratinocytes that had previously been stimulated by *P. acnes*-extracts. This could be an additional mechanism to those already described contributing to the anti-inflammatory action of zinc since stimulation of TLRs leads to secretion of various cytokines [86]. A study comparing the effect of oral zinc gluconate and minocycline hydrochloride, a topically applied antibiotic, came to the conclusion that minocycline was superior to zinc gluconate, but nevertheless both treatments were effective concerning inflammatory acne vulgaris. Zinc seemed to be more effective in females and in older patients. Adverse effects caused by zinc were moderate and mainly affected the gastrointestinal tract. Thus, zinc should be considered as alternative acne treatment [87].

Viral warts that were resistant to all forms of treatment showed a high rate of regression under oral zinc sulphate compared to placebo. Adverse reactions were mild and transient comprising gastrointestinal symptoms such as nausea and vomiting. Notably, serum zinc in patients suffering from viral warts was lower compared to controls. The effect of zinc supplementation on viral warts might be due to immunmodulatory activities of zinc or to the replacement of an underlying zinc deficiency [81].

4.2. Topical Application of Zinc

Products available for topical application of zinc include occlusive adhesive dressings, alginates, zinc-saline dressings, paste bandages and stockings [70]. Skin diseases which can be treated with topical zinc are, for instance, acne vulgaris, diaper dermatitis, seborrhoeic dermatitis and dandruff, psoriasis, hypertrophic scars and, as already discussed in section 2, chronic cutaneous ulcers [11,84]. Additionally, zinc is used in sunscreens in order to prevent photodamage [11].

Regarding acne vulgaris, a combination of 4% topical erythromycin, a systemic antibiotic, and 1.2% zinc acetate has been proven to be effective in acne treatment [88-90]. An *in vitro* study supporting the effectiveness of this combination therapy showed that zinc gluconate led to progressive reduction of resistance of *P. acnes* strains to erythromycin, which is an especially relevant finding considering the rising resistance rates of *P. acnes* to certain antibiotics [91].

Diaper dermatitis is an inflammation of the skin usually resulting in erythema, scaling and sometimes vesicles or erosions. Predisposing factors are increased skin wetness while wearing diapers, fecal enzymes and microorganisms [92]. A formula consisting of zinc oxide and petrolatum-based formulation led to reduction of irritant skin alterations frequently observed during use of diapers. Zinc oxide was shown to be delivered to the skin and in combination with petrolatum formulation it reduced erythema more markedly compared to petrolatum formulation alone or control [93]. This study clearly indicates the usefulness of topical zinc in prevention of severe diaper dermatitis. However, concerning the treatment of manifest diaper dermatitis eosin 2% solution had a significantly better outcome compared to zinc oxide (47%) paste since eosin led to at least partial healing in 17 out of 18 patients, whereas with zinc oxide treatment, it were 13 out of 18 patients [94]. This study still indicates the effectiveness of zinc oxide concerning treatment of diaper dermatitis, but its value might be even greater when used for prevention of this skin affection.

A very well-known application of zinc is the use of zinc pyrithione shampoo in order to fight dandruff or seborrhoeic dermatitis of the scalp, skin conditions resulting in scaling of the scalp with or without erythema. These disorders seem to be associated with the presence of yeasts of the genus *Malassezia* [84,95,96]. Several clinical studies could show that zinc pyrithione is an effective treatment concerning dandruff [84,96-99]. The reduction of PAS-positive microorganisms (yeasts) observed when applying zinc pyrithione shampoo suggests that the positive effect mediated by zinc pyrithione is based on reduction of *Malassezia* spp. [97]. This assumption could later be confirmed [84,100]. The thesis that zinc pyrithione might positively influence dandruff by suppressing DNA synthesis and cell proliferation as observed in *in vitro* experiments [101] could not be confirmed *in vivo* [102]. When zinc pyrithione was compared to ketoconazol, a fungicide, ketoconazol showed a significantly better outcome strongly supporting the idea that *Malassezia* spp. play a causative role in dandruff [99]. Nevertheless, zinc pyrithione can be regarded as safe and effective anti-dandruff therapy [99].

Concerning psoriasis, results of topical zinc treatment are not convincing [84]. Application of a topical spray preparation containing zinc pyrithione and subsequent analysis of changes in histopathology of a psoriatic plaque resulted in reduction of inflammatory cells, increase of epidermal apoptotic bodies and a rapid return to relatively normal skin within 14 days [103]. This finding implies a possible therapeutic role of zinc in psoriasis. However, zinc pyrithione does not seem to act synergistically with clobetasol propionate since no improvement of psoriatic conditions was registered when zinc pyrithione was added to clobetasol propionate [104]. Hence, the relevance of zinc pyrithione in the treatment of psoriasis is not clear and further studies are necessary.

One study examined the effect of topical zinc on hypertrophic scars and keloids in humans, which are due to excess scar formation [105,106]. In this uncontrolled study, zinc oxide applied in form of a zinc tape, led to reduction of itching, redness and the volume of the hypertrophic tissue. That these effects are mediated by zinc cannot be concluded, though, because a control treatment accounting for the effect of tape treatment without zinc is missing [106]. Support for the use of zinc in treatment of hypertrophic scars is provided by a recent study examining the potential of zinc oxide to prevent development of hypertrophic scars following full thickness skin excisions in rabbits compared to placebo. In this study topical zinc significantly reduced 6th week

clinical scar hypertrophy scores indicating a preventive effect regarding hypertrophic scars and keloids [107].

Finally, the use of zinc concerning the prevention of UV (ultraviolet) radiation-induced skin affections shall be shortly discussed as zinc is ingredient of many sunscreen products [11,108]. Zinc oxide is used as a physical sunscreen that protects the skin from UVA and UVB radiation by reflecting, scattering and absorbing UV radiation. In order to prevent whitening of the skin, nanoparticles are used more frequently instead of bigger zinc aggregates [108]. Apart from the function as a physical sunscreen, *in vitro* and *in vivo* experiments analyzing animals indicate that zinc might have an additional non-physical effect. For example, topical zinc prevented the development of sunburn cells in mouse epidermis even when applied after irradiation and it reduced the development of micronucleated cells induced by UVA and UVB radiation *in vitro* [109]. Furthermore, in human fibroblasts, zinc reduced DNA-strand breaks as well as apoptosis induced by UVA₁ radiation [110]. A very recent study showed that zinc pyrithione topically applied to mice prevented skin hyperplasia induced by UVB. This hyperplasia might be associated with skin photoaging and carcinogenesis. HIF (hypoxia-inducible factor)-1α, a factor supposed to be involved in epidermal homeostasis, is suppressed in epidermal overgrowth after UVB radiation. Zinc pyrithione was shown to prevent this suppression of HIF-1α indicating a possible mechanism, other than its physical properties, by which zinc might modulate skin reactions to UV light possibly preventing undesirable effects of UV radiation on skin [111].

A finding raising concern is the observation that small amounts of zinc oxide nanoparticles are absorbed through the skin and can be detected in blood and urine especially in females [112]. Whether, the absorption of small amounts of zinc might be harmful in any way, considering that sunscreen is supposed to be applied frequently and life-long, still has to be analyzed. Thus, zinc in sunscreen seems to be a topic that will be further discussed in the future.

5. Conclusion and Perspectives

The relation of zinc to human skin and skin disorders is manifold and still not completely understood. Although zinc has been used empirically already by Egyptians 3000 years ago [1,2], the first hint linking zinc with skin conditions was given when skin lesions of persons with zinc deficiency could be assigned to the lack of zinc [15,16]. Since then, many studies were conducted analyzing the role of zinc in various skin affections thus revealing the importance of zinc in wound healing, some possible underlying mechanisms, its antibacterial effect and potential therapeutic options regarding healing processes [70]. Furthermore, the association of zinc with several skin disorders has been examined over the last 40 years and the value of zinc as treatment option has been investigated. However, studies analyzing zinc as a therapeutic agent in diverse skin conditions such as acne vulgaris, diaper dermatitis, seborrhoeic dermatitis and dandruff, psoriasis and hypertrophic scars have produced contradictory results, indicating that the benefit of zinc treatment might only be present in certain populations using certain zinc products [11,84,113]. The dose of zinc as well as its form and vehicle used for topical application could have an impact on zinc effectiveness [4,34,55], as well as the zinc status of the patient [15,27]. Although reported adverse reactions to systemic and topical zinc were mostly restricted to mild or moderate

gastrointestinal symptoms [28,30,43,81,87], the absorption of zinc nanoparticles from sunscreen is critically observed because of the frequent use of sunscreen products, especially in sunny regions [112].

Thus, in the field of systemic and topical zinc application including adverse reactions, further research is necessary in order to define parameters that might predict a beneficial effect of zinc on skin disorders in order to optimize treatment strategies and to individually assign zinc treatment to those patients who are most likely to profit.

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27. Zinc and Eye Diseases

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Abstract. The eye, especially the retina and the underlying retinal pigment epithelium/choroid complex, contain high concentrations of zinc. Therefore, it is not surprising that several eye disorders are associated with altered zinc balance, and zinc supplementation has become a choice of treatment for diseases like age-related macular degeneration. Despite its importance in health and diseases of the eye it is still not well understood how zinc participates in cellular and molecular events and how zinc supplementation might be beneficial.

Keywords. Eye diseases, age-related macular degeneration, drusen, complement factor H, retinal pigment epithelium, retina, Bruch's membrane, choroid, endothelium.

Introduction

A decade ago, Grahn et al. [1] reaffirmed the sentiments of Karcioğlu [2] in 1982 that knowledge on zinc metabolism in the eye is fragmentary and quite confusing. Despite the widening research on zinc and extensive molecular works on zinc transporters only a few seminal works [3-5] have been published since 2001, therefore the statement of Karcioğlu still remains largely true today. In recent years, research in the field has gained momentum following the publication of a number of major population based studies looking at the role of nutrition in prevention of Age-related Macular Degeneration (AMD) the leading cause of blindness in western societies. Investigation of the association between zinc in the diet and clinical eye manifestations was not a new concept but the findings of AREDS group [6], the Blue Mountain Eye Study [7], the Beaver Dam Eye study [8] and the Rotterdam Eye Study [9] have captured the interest of the public and clinicians, highlighting a vital need for better understanding of the molecular mechanisms involved. Other factors behind the intensified interest in the zinc biology of the eye is our increasing understanding of the functions of zinc in the brain and in particular in association with Alzheimer's disease [10] as well as a better understanding of zinc transporting [11] and buffering in cells [12]. In this chapter we look at how zinc is or may be involved in different eye diseases and summarize our current knowledge of zinc biology in the eye without reproducing previous excellent summaries [1, 3].

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1. Consequences of Zinc Deficiency and Overload in the Eye

1.1. Zinc Deficiency

Several studies have demonstrated manifestations of zinc deficiency within the eye. Some of the effects can be very subtle and misdiagnosed as relating to other eye diseases [1]. Most of these however, have their pathogenesis linked by decreased serum zinc, occasionally reduced tissue zinc concentrations or by improvement after zinc supplementation [1]. The most interesting observations have risen from studies of patients with systemic conditions which lead to zinc deficiency. One such disorder is acrodermatitis enteropathica that is a rare early childhood disease, with multiple systemic manifestations caused by an abnormality in zinc metabolism. The ocular abnormalities observed in these patients include blepharitis, photophobia, conjunctivitis, corneal opacities, cataracts, superficial punctate opacities, nebulous subepithelial opacities and linear corneal erosions [1, 13-17]. Gene expression studies in acrodermatitis enteropathica suggest that there are mutations affecting mRNA of ZIP4, a zinc transport protein, which results in decreased zinc absorption [18]. Additionally Cherry-red maculopathy and visual impairment have been reported in men with Crohn's disease that can be treated in some cases by zinc supplementation [19, 20]. Increased fecal zinc and decreased granulocyte zinc have been described with desferrioxamine use in thalassaemia cases [21]. Patients receiving long-term total parenteral nutrition have been reported with altered visual function and decreased plasma zinc concentrations [22]. Similarly, abnormal dark adaptation and diminished scotopic retinal responses are associated with zinc deficiency in alcoholism and hepatic cirrhosis. These conditions often respond to zinc supplementation, but also may require supplemental vitamin A [1, 23-27]. Here we also describe some of the other ocular manifestations of zinc deficiency as shown in animals and human experiments.

Studies in rats have been particularly useful in demonstrating that zinc is essential for ocular development. Rats reared on diets severely deficient of zinc during gestation, had pups with optic cup invagination failure, colobomata, retinal dysplasia and occasionally anophthalmia [1, 28]. Gottschall-Pass et al. [1, 29] have reported retinal dysplasia and depression of electroretinograms in pups born to rats fed a diet marginally zinc and taurine deficient throughout gestation and postnatal life until 7.5-8.5 weeks of age [1, 29, 30]. Exclusively post natal zinc deficiency in these rats failed to result in any morphological changes despite depression of electroretinogram and oscillatory potentials. In other studies weaning rats maintained on a severely zinc deficient diet for seven weeks had degeneration of outer segments and osmiophilic inclusion bodies in the retinal pigment epithelium (RPE), visualized by electron microscopy [1, 31-33].

1.2. Zinc Overload

Zinc toxicity in general is not a common or major health problem and there are only anecdotal reports of severe toxicity [34]. However, excess zinc have been shown to induce or facilitate cell injury in the eye that has led to its description as a "double edged sword" [3]. Isolated rabbit retinas exposed to 500 μ M or more of zinc led to the release of GABA and NMDA that could be completely prevented by inclusion of the zinc chelator diethyldithiocarbamate (500 μ M) in the incubation medium [3]. High levels of zinc can activate several intracellular cascades which may ultimately cause

neuronal destruction. Most important of these appear to be activation of reactive oxygen species (through a mechanism not clearly understood) and interruption of energy production and blockade of sodium/potassium-ATPase [3]. High amounts of zinc have also been shown to inactivate the antioxidant enzymes glutathione peroxidase and glutathione reductase [35]. Furthermore, zinc can alter the conformation of neurotrophins [36] and alter the survival of retinal cells [37, 38].

2. Distribution of Zinc in the Eye

The ocular tissue has an unusually high concentration of zinc compared to other tissues [39] with the highest amount of this concentrated in the RPE and choroid. The descending order of zinc levels in other ocular tissue parts is ciliary body, iris, optic nerve, sclera, cornea and the lens [1, 2].

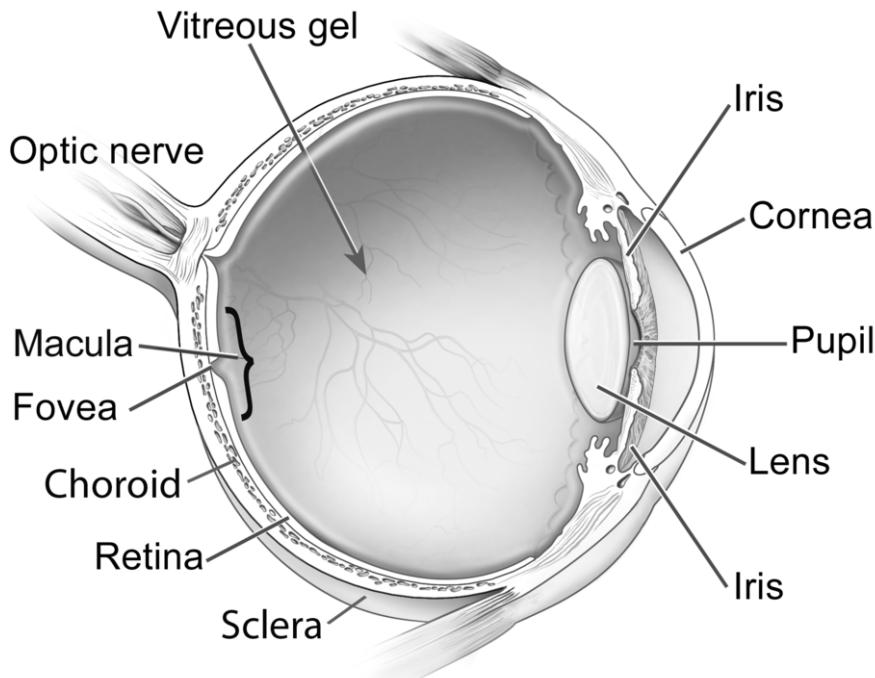


Figure 1. Schematic diagram of the structure of the human eye. (Reproduced from National Eye Institute, National Institutes of Health, Ref#: NEA09.)

2.1. Cornea and Conjunctiva

The cornea is the eye's outermost layer directly exposed to the environment. It shields the rest of the eye, focuses the light and contributes to the eye's total focusing power. It contains no blood vessels so it receives its nourishment from the tears and aqueous humor. The cornea must remain transparent to refract light properly. For good vision, all 5 layers of the cornea must be free of any cloudy or opaque areas. Although the cornea is not rich in zinc in comparison with other ocular tissues [40], its zinc content plays an important role in maintaining opacity by regulating wound healing [41-43] through a multitude of zinc dependent enzymes that play a critical role in wound healing and immune response in the cornea [2, 44, 45].

In studies of rats given zinc deficient diets, examination of the corneal and conjunctival epithelium revealed scarce microvilli and microplicae (characteristic ridge-like folds of plasmalemma) [46, 47]. The conjunctival epithelium also had reduced number of goblet cells. These changes can induce ocular surface disturbances due to breakdown of the tear film and will affect the defense mechanism of the ocular surface against infection [48]. This explains the reduced immunity of the cornea in zinc deficiency [48]. The tissue abnormalities can all be reversed by zinc supplementation [48]. Other studies have reported that zinc-deficiency can lead to conjunctivitis, ocular discharge, keratitis [49] and recalcitrant corneal ulcers [50]. The observed changes seen in the cornea and conjunctiva in zinc deficiency may explain the ocular surface symptoms such as foreign body sensation and tearing [2].

2.2. Lens

The lens focuses light onto the retina at the back of the eye, where an image is recorded. Therefore, it is very important that the lens is free of optical disturbance. The lens is made mostly of water and precisely arranged proteins. The maintenance of this arrangement is a key factor for clear vision but as we age, proteins may clump together and start to cloud the lens leading to cataract formation.

The lens contains a substantial amount of zinc [39, 51] that plays an important role in regulating enzyme systems [52, 53]. It has been suggested that zinc deficiency plays a crucial role in the etiology and development of cataracts in animals [54-57] as well as in human [15], however the evidence is somewhat conflicting [2, 58-60]. It has been observed that the cataractous changes are secondary to disturbed glucose utilization through disregulation of zinc metalloenzyme activity in the lens [53]. Administration of zinc can reverse this anomaly and prolonged zinc supplementation was suggested for prophylaxis and treatment of cataracts [53]. However, no beneficial effect of zinc supplements on development or progression of cataract was observed in a population based study [61]. Loss of copper-zinc superoxide dismutase activity [62, 63], altered sodium/potassium-ATPase activity and electrolyte transport [64] are all associated with cataract formation.

Goldstein et al. reported the pathologic accumulation of amyloid beta ($\text{A}\beta$) in lenses of Alzheimer's disease (AD) patients [65]. In these lenses $\text{A}\beta$ accumulates as electron-dense aggregates leading to supranuclear opacities that ultimately progress to distinctly different cataracts from age-related nuclear cataracts [65]. Similarly, amyloid accumulation was also shown in subjects with Down's syndrome [66] suggesting that processes similar to plaque formation are involved. Given the well documented role zinc plays in plaque formation in Alzheimer's disease [67] one could speculate that

zinc within the lens tissue is involved in this process, and recent experiments support this speculation [68].

2.3. Iris

The iris controls the amount of light that enters the eye. Its color partly depends on the amount of pigment. As melanin binds metal ions such as zinc [69-71] dark irises should contain higher levels of zinc [72]. Kokkinou et al. [73] compared irises from brown eyes with those of blue eyes and showed a significant difference in melanocytes. Blue irises also contain significantly fewer melanosomes [74] hence less zinc [73]. In addition, significantly higher zinc uptake into brown irises was seen [73]. This observation is similar to that of Newsome et al. [75] who have suggested that melanin in the RPE influences the uptake and accumulation of zinc. As melanin [76] and zinc [77] content of the tissue reduce with age, so does the antioxidant capacity as a consequence [73]. This latter finding may be important for combating photo oxidative damage; however, there is no other known iris associated disease of zinc homeostasis. A significant association between light iris colour, fundus pigmentation and AMD has been suggested [78], but the suggestion that iris pigmentation is associated with risk of developing AMD has remained controversial [73].

2.4. Vitreous

The vitreous fills the space between the lens and the retina. It contains very few cells and no blood vessels, and 98-99% of its volume is water with salts and sugars. The rest is made of a network of collagen type II fibers, hyaluronic acid and a small amount of diverse proteins [79, 80]. The vitreous is considered a repository for metabolites and biochemical waste for retinal metabolism [81]. Zinc content in the vitreous varies, depending on the species used [1]. Little is known about the role for vitreal zinc but zinc levels have been shown to be affected in the vitreous in cataract [82], diabetes [83] and alcoholism [84]. One important role for zinc in the vitreous can perhaps be demonstrated in Eales's disease [85, 86] where zinc has been linked to protection against oxidative stress [87, 88]. Potentially, zinc could be an important mediator of the immune system in the vitreous or affect the collagen in vitreous, similar to that of the cornea [44, 45].

2.5. Optic Nerve

Zinc is thought to be essential for normal optic nerve function, from the observations of optic neuritis, optic atrophy and neuropathy in patients with liver cirrhosis [89, 90], chronic alcoholism [91-93], Crohn's disease [94, 95], acrodermatitis enteropathica [18, 96] and patients on drugs such as ethambutol [97], disulfiram and penicillamine [98, 99].

In zinc deficiency the number of myelinated axons of the optic nerve has been shown to be significantly reduced and the myelin sheaths are significantly thinner in rats. Return to a zinc sufficient diet does not aid recovery and the axons continue to degenerate after resumption of a normal diet [47]. Reduction in the number of myelinated axons has also been observed in studies of rhesus monkeys treated with ethambutol [100]. One suggested route through which zinc can affect myelin is through copper-zinc superoxide dismutase and increase in lipid peroxidation [47, 101]. These

data suggest that once degeneration of the optic nerve had commenced there can be no recovery, hence zinc deficiency may lead to permanent visual impairment.

2.6. *Tapetum Lucidum*

Although the tapetum lucidum is absent from human eyes [102] it deserves a mention in this chapter because of its high concentration of zinc (7.2% of the total dry weight of a dog's eye [2]) and its important role in low level vision in animals. The tapetum lucidum is a modified choroidal structure which reflects light off its shiny surface back through the retina, and enhances low-level illumination. It is one of the most extensively studied of all zinc containing parts of the eye [2]. In the cat eye, the tapetal cells contain a zinc protein high in cystine [103, 104]. The zinc-cystine complex has been shown to exhibit photoelectric properties and this amplifies the response to low level illumination [105]. Microprobe analysis of the tapetum has demonstrated that zinc is located in specialized intracellular organelles known as tapetal rodlets [106]. An inherited tapetal abnormality has been reported in the dog and cat, in which the tapetal rods contain significantly less zinc than those of control animals with clinical manifestations including a lack of tapetal development [107-109].

2.7. *Sclera*

The sclera is a dense fibrous tissue that can withstand the expansive forces of the intraocular pressure and protects the eye from external trauma. Its overall role is to support the visual apparatus but it can also influence the refractive properties of the eye. The function of zinc in sclera is likely to be associated with the regulation of the sclera extracellular matrix [110]. Zinc affects collagen [44, 45] and metalloproteinases secreted by scleral fibroblasts [111, 112] and therefore may play an important role in the dynamic remodeling of the sclera [113].

2.8. *Retina*

The retina-choroid complex contains the highest concentration of zinc in the eye as measured using a wide variety of techniques in different species including human [39, 45, 110, 114-123]. The majority of this zinc is tightly bound to proteins, but a proportion is histochemically reactive as shown by using autometallography [3, 124-127], dithizone [128] and fluorescent zinc sensors [4, 129-132]. This reactive pool is referred to as "free", "labile", "readily releasable" or "exchangeable" zinc. The definition of this pool of zinc is widely discussed [12, 133]; we will refer to this pool as exchangeable zinc to highlight the dynamic nature of it. The distribution of total and exchangeable zinc in the retina/choroid complex is depicted in Figure 2.

Electron microscopic examination of autometallographic localization has shown zinc to be localized to the Golgi apparatus of ganglion cells, the neuronal processes of the outer and inner plexiform layers, the Golgi apparatus of the horizontal and amicrine cells as well as other cell types in the inner nuclear layer, the nucleus of the photoreceptors and Muller cell processes in the outer nuclear layer [125, 126]. Muller cell localization of zinc had also been shown in other studies [134, 135]. Depolarization of the retina can induce zinc release at the plexiform layers [4] which provides the first evidence for the hypothesized neuro modulator role for zinc in the retina [5, 136].

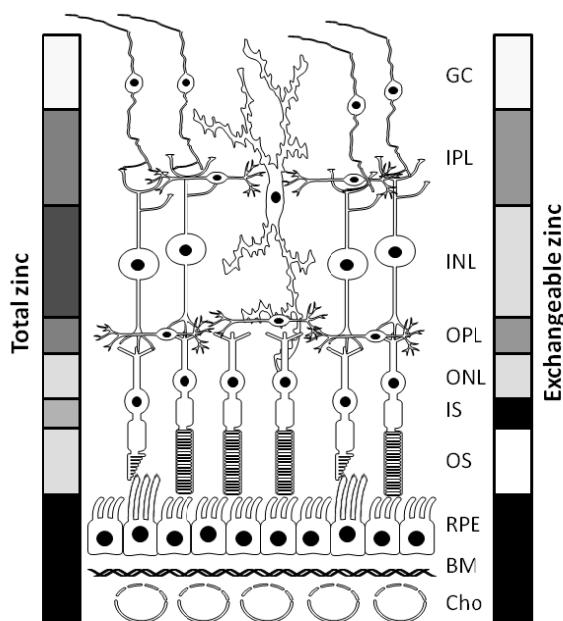


Figure 2. A simplified diagram depicting the cellular organization of the retina/RPE/choroid complex: Cho, choroid; BM, Bruch's membrane; RPE, retinal pigment epithelium; OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer and GC, ganglion cell layer. The shaded bar in the right represent exchangeable zinc levels in the different layers based on [3, 124-127]. The shaded bar on the left represents total zinc levels based on [39, 45, 110, 114-123]. White represents lowest and black the highest concentration.

Interestingly no outer segment labeling was observed by Akagi et al [125] despite other reports showing the presence of zinc in outer segments using dithizone [128] autometallography [127] and TSQ [129] or zinpyr-1 fluorescence [131]. The availability of exchangeable zinc is very important for outer segment functions. Pålsgård et al [120] showed that total zinc levels are substantially increased in light adapted outer segments. Zinc is a requirement for the stabilization of discs in outer segments [137] probably through the stabilization of rhodopsin [138, 139]. However, low affinity zinc binding to specific histidine sites can lead to de-stabilization of rhodopsin [140] and decreased thermal stability [141] suggesting that rhodopsin might be less stable in light than in dark. This zinc-induced de-stabilization of rhodopsin might be especially relevant in diseases such as retinitis pigmentosa which is associated with a Pro23His mutation [142] that introduces further zinc-induced instability of rhodopsin [140]. Interestingly retinitis pigmentosa is also associated with systemic zinc deficiency [143]. How all this might lead to a zinc-based intervention strategy in this devastating eye disease [140] will require further consideration. Outer segment zinc content can also affect dark adaptation [144] through the conversion of vitamin A (retinol) to retinal by the zinc-dependent alcohol dehydrogenase [145, 146] and this can be clinically manipulated by zinc supplementation [23]. Zinc may also be involved in dark adaptation by affecting the synthesis or release of retinol-binding proteins in the

liver [147]. Where and how the reported increase in total zinc in the outer segment [120] originates from will be an important factor to determine.

RPE has very high concentrations of zinc which can decrease during periods of zinc deficiency [72] but are conserved in marginal zinc deficiency [118]. Zinc content and retention is regulated, at least partly, by pigmentation of the RPE [73, 75]. As melanin synthesis and melanosome formation appears to be regulated by zinc [148] this is probably not surprising. The RPE is also rich in exchangeable zinc [125, 127, 129] localized to the Golgi apparatus [125] melanosomes and lysosomes [3, 117, 149, 150]. Newsome et al. [75, 151] have demonstrated an active uptake and prolonged retention of zinc by RPE. The RPE in the macula contains significantly less zinc than the peripheral retina although zinc levels are also affected by aging [152]. Zinc deficiency not only resulted in a reduction in metallothionein concentration, decreased cell proliferation, reduced protein production and decreased activity of catalase, alkaline phosphatase and alpha-mannosidase in RPE [153, 154] but the cells were also more susceptible to oxidative insult [155]. Based on these data zinc deficiency was proposed to be involved in age-related macular degeneration (AMD) [61, 156] (for further details on zinc and AMD see paragraph 4).

The extracellular matrix between the RPE and the choroidal microcapillaries, called Bruch's membrane, contains a substantial amount of zinc especially in eyes with AMD [132]. As some of this zinc is in the exchangeable form [132], it is possible that there is an extracellular zinc milieu that requires active control for the regulation of extracellular proteins involved in immune response [157] or in the remodeling of the extracellular matrix by metalloproteinases [158].

The highest concentration of zinc in the eye has been localized to the choroid [121, 122]. As this is a highly pigmented layer, it is likely that pigmentary zinc contributes to this enrichment. However, relatively little is known about the role and function of zinc in the choroid, other than it appears to accumulate with increasing age [121] and may contribute to the developing zinc deficiency in the aged eye, although this notion is still controversial [123].

3. Distribution of Zinc Transporters in the Eye

In most tissues, the amount of exchangeable zinc is maintained within a narrow concentration range. When exchangable zinc levels are high, cells will sequester excess zinc, and when zinc levels are low, cells will increase zinc influx. This illustrates the importance of understanding how the levels of zinc are regulated. The majority of the changes in zinc levels are mediated through 24 transmembrane proteins (10 zinc efflux transporters called ZnT 1-10 and 14 influx transporters called ZIP1-14) encoded for by two solute-linked carrier (SLC) gene families, SLC30 and SLC39, respectively [159] (for details see chapter 8). Despite the perceived importance of appropriate zinc homeostasis in ocular tissues, relatively little is known about where zinc transporters are localized, and how they are involved in the normal and pathological function of the eye.

3.1. Zinc Transporters in the Retinal Pigment Epithelium.

Most attention has been focused on how zinc homeostasis is regulated in the RPE cells. This is due to the exceptionally high zinc concentration in these cells and the emerging

importance of zinc in maintaining the right environment for photoreceptor function and therefore light processing. Leung et al [160] has determined the expression levels of 23 zinc transporters in ARPE-19 cells, a widely used immortalized human cell line to study RPE function [161], and primary cultures of RPE from human fetus and adults. They showed that mRNAs for 21 of 23 transporters were expressed in ARPE-19, 20 of 23 in fetal human RPE, and 16 of 23 in the adult RPE (Figure 3). ZnT5 was not detected in any of the RPE samples analyzed. The transporters ZnT2, ZnT8, ZIP2, ZIP6, ZIP9, and ZIP11 were detected in ARPE-19 and the fetal RPE cells but not in the adult cells. ZnT3 and ZIP5 were expressed in ARPE-19 and the adult human RPE but not in the fetal cells. ZIP12 was the only transporter found in the primary human RPE cultures but not in the cell line. Rezaei et al [162] determined the expression of ZIP1-14 in ARPE-19 cells. Twelve of the 14 Zip family transporters were detected in their assay. They found that the ZIP4 and ZIP12 RNA were absent in ARPE-19 cells. We have also measured the expression of all 24 known zinc transporters in ARPE-19 cells (Figure 3). We found 6 of the 10 ZnTs and 12 of the 14 ZIPs to be present in ARPE-19 cells. We did not observe expression of ZnT2,3,8 and 10 and ZIP2 and ZIP12 in these cells. Differences between the three laboratories could simply be attributed to differences in experimental conditions (i.e. batches of cells and/or passage numbers of ARPE-19 cells) or differences in PCR primer design, but nevertheless these data suggest that zinc transporter expression is probably dynamically regulated in RPE cells and that this may play a role in the degeneration of the retina.

Due to the paucity of factual information, our current knowledge on zinc transporters has been summarized in a representative diagram containing information from the above mentioned studies as well as information gathered from the literature (Figure 4). ZnTs usually transport zinc from the cytosol into the extracellular space or into intracellular organelles (labelled by black arrows on Figure 4). The ubiquitous ZnT1 protein, that was the first to be discovered amongst plasma membrane zinc transporters [163], was highly expressed in cultured RPE cells as well as in micro dissected human RPE [164] suggesting that ZnT1 is probably the main zinc efflux mediator in RPE cells. Based on studies on other cells [165, 166], ZnT1 is expected to be both on the apical as well as the basement membrane of RPE cells, and Leung et al [160] reported its perinuclear localization on permeabilized RPE cells. ZnT2 is an intracellular organelle associated protein [167] usually sequestering zinc into lysosomes [168, 169] and mitochondria [170]. ZnT2 might be involved in the processing of phagocytosed photoreceptor outer segments and the degradation of opsins [140] hence playing a very important role in the RPE. Furthermore, ZnT2 is able to respond to the changing environment [160, 171] which may explain why it was not observed by us but expressed and regulated in the study of Leung et al [160]. ZnT3 is usually associated with synaptic vesicles but has also been shown to be present in non neuronal cells [172]. Leung et al [160] found not only the RNA but also the protein for ZnT3 in RPE cells and they reported a significant up regulation of mRNA levels following pigment epithelial-derived factor (PEDF) treatment. ZnT3 has also been found on the apical part of the choroid epithelial cells in brain [168], mostly associated with apical microvilli and apical vesicle. Hence if ZnT3 was present in RPE cells then it would be expected to be associated with vesicles around the apical plasma membrane. However, ZnT3 expression will need further verification as we were unable to confirm this observation. ZnT4 expression has been found in all types of RPE cell cultures (Figure 3) despite the fact that Expressed Sequence Tags (EST) data suggests that ZnT4 is absent in the eye [173]. This transporter has usually been associated with intracellular vesicles in the

trans-Golgi network [171], hence vesicular location is expected in RPE cells. ZnT5 expression was absent in one report [160] but present in the eye based on Expressed Sequence Tags (EST) database [173] and it was observed by us in ARPE-19 cells. This transporter is reported to be involved in vesicular zinc loading as well as associated with apical membranes, yet the direction of zinc transport through this transporter is not clear probably because of alternative splicing [174-176]. The expression of ZnT6,7 and 9 was observed both by us and Leung et al [160]. ZnT6 and 7 are involved in translocation of cytoplasmic zinc into the Golgi network [177], while ZnT9 is ubiquitously distributed in cells it was thought to be involved also in zinc transport into the nucleus [178, 179]. As intracellular immunoreactivity of ZnT7 in RPE is very high, transporting zinc into the Golgi might be important for appropriate RPE function. We did not observe ZnT8 expression in our experiments but it was highly expressed, and developmentally regulated in another study [160]. ZnT8 is normally associated with vesicular loading of zinc in the pancreas, breast and lung [173], hence if it is present in the RPE then ZnT8 might be involved in the endosomal loading of zinc.

	hf RPE ^a	ha RPE ^a	ARPE-19 ^a	ARPE-19 ^b	ARPE-19 ^c
ZnT1	Black	Gray	Black	ND	Gray
ZnT2	Gray	Gray	Gray	ND	
ZnT3		Gray	Gray	ND	
ZnT4	Gray	Gray	Gray	ND	Gray
ZnT5				ND	Black
ZnT6	Black			ND	Gray
ZnT7	Gray	Gray		ND	
ZnT8	Black		Gray	ND	
ZnT9	Black	Gray	Black	ND	Black
ZnT10	ND	ND	ND	ND	
ZIP1	Black				
ZIP2	Black	White	Gray	Gray	White
ZIP3		Gray	Gray	Black	Gray
ZIP4	Gray	White	White	White	Black
ZIP5	White	White	Gray	White	Gray
ZIP6	Black	White	Black	Black	Gray
ZIP7		Gray	Black	Black	Gray
ZIP8			Black	Gray	Gray
ZIP9			Black	Gray	Gray
ZIP10		Gray	Black	Gray	Gray
ZIP11	Gray	White	Gray	White	White
ZIP12	Gray	Gray	White	White	Black
ZIP13	Gray	Gray	Black	Gray	
ZIP14	Gray	Gray	Black	Gray	Gray

Figure 3. Summary of zinc transporter expression in the eye based on the work of three different laboratories: a [160]; b [162]; c (Cahyadi, Berzegar-Befroei and Lengyel, unpublished). Black represents high, gray low and white no expression of a gene based on qRT-PCR experiments. Expression levels are compared to selected housekeeping genes. Abbreviations: RPE, retinal pigment epithelium; hf, human fetal; ha human adult; ND, not determined.

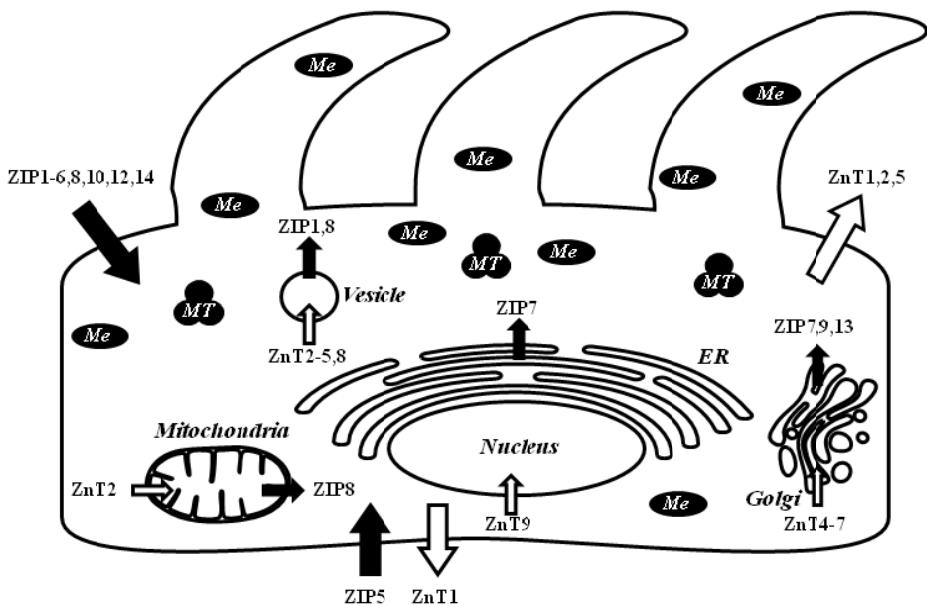


Figure 4. Zinc transporters in RPE. Based on literature information in other cell types and gene expression studies on RPE cells this figure summarizes our understanding of where zinc transporters may be located in RPE cells and includes potential zinc buffering or muffling compartments. For simplicity, on this figure endosomes, lysosomes, and secretory vesicles are labeled as “Vesicles” despite their vastly different function. Abbreviations: Me, melanosomes; MT metallothionein; ER, endoplasmic reticulum. Arrows represent the direction of zinc transport: black for Zips and white for ZnTs.

The ZIP family of zinc transporters promotes the transport of zinc from the extracellular environment or from cellular organelles into the cytoplasm (labeled by black arrows on Figure 4). Relatively little is known about these transporters. ZIP1-6,8,12 and 14 are usually plasma membrane located and likely to transport zinc into RPE cells. However, ZIP7 is localized to the ER and Golgi, ZIP8 is present on vesicles and mitochondria and ZIP13 regulates zinc levels in the Golgi (see recent summary in [180]). Although ZIP1 localizes to the plasma membrane it is also able to shuttle into intracellular vesicles under excessive zinc condition [181]. Expression of ZIP2 has been shown to be regulated by extracellular zinc levels in RPE cells that, in turn, will increase the levels of gamma-glutamyl cysteine ligase (a rate-limiting enzyme in glutathione synthesis) and glutathione to provide better protection against oxidative stress [162, 182]. The expression of ZIP2 appears to be developmentally regulated and can be altered by neurotropic factors in RPE cells [160] therefore it might be very important for normal and pathological processes in the eye. ZIP12 is probably the least studied zinc transporter protein. Its gene expression is reported to be localized to the retina, kidney and brain according to the EST database (<http://www.ncbi.nlm.nih.gov/nucest>). Leung et al. [160] reported that the expression of *SLC39A12* was found in primary cultures of human fetal RPE, as well as human adult RPE but not in ARPE-19 cells. Rezaei et al [162] and our work (Figure 3) confirms the lack of mRNA for ZIP12 in ARPE-19 cells while Booij et al [164] have confirmed that ZIP12 is expressed in RPE, and in fact is one of the RPE specific genes.

Given the important role for zinc in RPE cells the understanding of transporting mechanism will be essential to understand how this highly specialized cell plays a role in normal as well as pathological events in the eye.

3.2. Zinc Transporters in the Retina.

Relatively little is known about zinc transporters in other parts of the eye. Preliminary quantitative RT-PCR data suggests that endothelial cells express 6 out of the 10 ZnTs and 12 out of the 14 Zips (Cahyadi, Berzegar-Befroei and Lengyel, unpublished). Muller cells express 8 out of the 10 ZnTs and 11 out of the 14 Zips, while primary retinal ganglion cells express 9 out of the 10 ZnTs and 13 out of the 14 Zips (Cahyadi, Berzegar-Befroei and Lengyel, unpublished). Redenti et al [134] found ZnT3 protein in several layers of the retina, namely the inner segments and outer limiting membrane of photoreceptors, the inner nuclear layer and outer plexiform layer and the strongest labeling was present in both ganglion and Muller cells. This associates ZnT3 and therefore zinc with neuronal transmission [172]. The only other transporter whose immunolabeling has been reported in the literature is ZnT7 [127]. Immunolabeling for ZnT7 is present in optic nerve fibers, ganglion cells, inner and outer plexiform layers, horizontal and amacrine cells and the photoreceptor outer segments [127], suggesting that ZnT7 plays a wide ranging role in the retina.

3.3. Eye Phenotype in Zinc Transporter Knockout Animals.

The role of zinc and zinc-dependent mechanisms in development [183] indicated that appropriate zinc homeostasis might be important for normal eye development. In support of this, the examination of eye phenotypes of transgenic animals showed that an array of developmental abnormalities is associated with the lack of zinc transporter genes. For example, in ZIP4 knockout mice, exencephalia, severe growth retardation, and hydrocephaly, was accompanied by unilateral or bilateral anophthalmia. The manifestations of these could be exacerbated with zinc deficiency and ameliorated by zinc supplementation in heterozygous but not in homozygous embryos [184]. Fukada et al reported that ZIP13 is crucial in connective tissue development. Zip13 knockout caused sunken eyes which were also associated with down slanting palpebral fissures [185]. The abnormalities were attributed to a decrease in dermal collagen fibril sizes and thinning of corneal stroma. ZIP13 knockout effects also extended to improper osteogenesis and craniofacial tissue development. Based on the limited information it is clear that zinc plays an important role in eye development.

4. Zinc and Age-Related Macular Degeneration.

Age-related macular degeneration (AMD) affects about one quarter of people over the age of 65 years [186] and late stage disease accounts for approximately 50% of legal blindness in Europe and North America [187, 188]. The etiology of AMD is not well understood and there are no medical interventions that could prevent the incidence or progression from the asymptomatic early stage to end-stage disease with visual loss. There are two forms of end stage disease. In geographic atrophy or “dry” AMD the RPE cells slowly degenerate and may atrophy completely, a progression that takes many years before blindness develops. If the integrity of Bruch’s membrane is broken

choroidal neovascularisation develops. This aggressive “wet” form of the disease progresses rapidly and leads to blindness in about 10% of the AMD sufferers. Wet AMD can be treated with some success [189].

A key feature of both forms of AMD is the presence of extra-cellular deposits between the choroid and the RPE [190]. These deposits vary in size and have been classified in a number of different ways [191-194]. Clinically the term drusen is used to monitor progression of visual loss, as the other types of deposits are not readily visualized. Small drusen may not always be associated with risk of visual loss. However, multiple large drusen, located in the macular region, increase the risk of AMD. Drusen accumulation occurs naturally with age and an individual druse has the capacity to distort and rupture through the RPE and pushing into the neural retina [194]. Hence, development and progression of sub-RPE deposit formation are likely to be a key factor in AMD pathogenesis. Understanding how such deposits are formed is key to understand this devastating eye condition [195]. The composition of sub-RPE deposits are very complex [196, 197]. They contain proteins and lipids as well as anomalous deposits of zinc, some of which is in the exchangeable (ionic or loosely protein bound) form [132]. One of the major questions that needs to be addressed is the origin of the millimolar zinc in the Bruch’s membrane (the extracellular matrix in which the sub-RPE deposits are formed).

4.1. Zinc and the Cellular and Molecular Events Associated with AMD

Like any other cells, the RPE can be damaged by too much or too little zinc [198, 199]. Newsome et al. demonstrated that levels of zinc are reduced in human eyes with signs of AMD [200]. This was proposed to lead to increased oxidative stress [155], deficits in phagocytic and lysosomal functions [169, 201], macromolecule synthesis- and caspase-dependent apoptosis [198], increased photic injury [186] and UV-induced DNA damage [202] in the RPE. This short and probably incomplete list shows that changes in RPE zinc may potentially play a multitude of roles in the development of AMD but does not provide an answer to where the millimolar zinc levels of zinc in the Bruch’s membrane are derived from. However, we did not consider that the RPE has very high concentrations of intracellular total zinc (see earlier in Figure 2). In addition, one the RPE’s function is the phagocytosis and processing of the zinc-rich photoreceptor outer segments, potentially enriching the RPE zinc content further. As RPE damage is thought to be the precursor for the development and progression of AMD [186] abnormal zinc release from RPE may occur as the consequence the damages highlighted above. The choroid is also rich in zinc and changes associated with AMD here [203] may also contribute to the accumulation of zinc in the Bruch’s membrane. These suggest that buffering zinc in the Bruch’s membrane could be important in mediating sub-RPE deposit formation and hence the development of AMD.

4.2. Zinc, Complement Factor H and AMD

Possibly the most interesting molecule in relation to sub-RPE deposit formation and AMD is complement factor H (CFH) and its Tyr402His mutant form [204-208]. It was shown almost 30 years ago that millimolar concentrations of zinc induced the oligomerisation of CFH and rendered it inactive in the test tube [209, 210]. Later it was found that there was no need for millimolar extracellular zinc levels to trigger oligomerisation and inhibition of CFH. Large oligomers were formed in the test tube at

higher than 20 μM zinc and by 200 μM zinc >85% of CFH is in oligomeric and fully inhibited form [157]. Could similar oligomerization occur in the Bruch's membrane? It has been shown that inactivation of CFH and the uncontrolled activation of the alternative pathway, resulting in secondary C3 deficiency, is part of the pathological process leading to AMD [211]. In addition, Hageman et al [205] provided evidence that CFH, together with C3b/iC3b, membrane attack complex and C5b-9, is a constituent of sub-RPE deposits. Therefore, the potential to release exceptionally high levels of zinc from the RPE through injury to this cell layer and the fact that the Bruch's membrane contain high concentrations of zinc in AMD [132] zinc could potentially induce the kind of pathological protein aggregation mentioned above. As oligomerized CFH will have attenuated complement inhibitor function, the RPE and the choroid will be at sustained risk for alternative pathway-mediated complement attack. Within the complement system zinc can affect more than just CFH. Zinc can bind to a number of complement proteins [212] and affect complement activity in several different ways [213-216]. As inflammation has been suggested to be the major pathological cause underlying AMD [205] these data make sense. Whether the Tyr402His mutation will serve as an additional zinc binding site requires experimental proof. Modeling studies [217] and our preliminary experiments on purified CFH support this idea [218].

4.3. Zinc to Treat AMD

As AMD has been associated with reduced levels of zinc [200], supplementation with zinc became one of the most widely used intervention strategies to combat the disease. This is a popular option as zinc supplements can be purchased without prescription in most countries. However, zinc supplementation trials and epidemiological studies have produced conflicting results [219-221]. In addition, high dose dietary supplementation had been shown to cause copper deficiency, suppress the immune system, increase the risk for metastatic prostate cancer and impair behavior (for recent review see [222]) and cause urinary complications [223]. Because of these it was suggested that supplementation probably should be replaced with a healthy balanced diet that provides the necessary vitamins and micronutrients the body needs [9]. Nevertheless, the idea that restoring zinc balance through diet or supplementation may protect against AMD provides an interesting and potentially inexpensive intervention strategy and trials are in progress to refine this concept [224]. However, we do not know at what stage the protective effects of zinc may be important, or the potential negative interactions with genetic and/or other risk factors starts. Therefore, the role of zinc in the pathogenesis of AMD needs to be thoroughly investigated.

5. Conclusion and Perspectives

A high concentration of exchangeable zinc is toxic to cells. It is therefore imperative that the very high concentration of zinc in ocular tissues, especially in the retina/choroid complex, is appropriately buffered to limit its concentration to physiologically acceptable intracellular levels [225]. Cytosolic zinc binding proteins can buffer intracellular zinc by direct binding to it, shuttling it into internal stores or removing it from the cells all together [12]. The identities of these molecules may hold the key of combating the many eye problems described above. It is well established that metallothioneins are highly but variably expressed in the eye [152, 226] and it is

likely that they can both bind and release zinc during oxidative stress or hypoxia [133]. Melanosomes may also serve as zinc buffers and/or stores in the RPE and the choroid [72, 117, 149]. Rhodopsin in the zinc-rich outer segments of photoreceptors may serve as zinc buffers [227] responding to changes in light levels [115, 140, 227]. Given the important role zinc and other trace metal plays in the degenerative processes in the eye, discovering the metallome of the eye may prove to be essential in combating eye diseases.

Few tissues in the body have total zinc concentrations high enough to release biologically reactive zinc in the range of 20–200 µM but the RPE choroid complex might be one, making this complex a potential target for pharmacological intervention. Zinc buffering-based therapies represent an entirely new approach to pharmacology [228]. Exchangeable zinc can be selectively removed from the plaques in Alzheimer's disease by compounds that are relatively weak zinc binders [229]. Such compounds are best described as zinc buffers, because they do not strip zinc from proteins, but merely stabilize the concentration of exchangeable zinc in the milieu. Given the important role for zinc in normal visual processing and its presumed involvement in the degeneration of the retina [3], the goal appears to be the restoration of optimal zinc balance in the eye which may slow the progression or even prevent the development of AMD.

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III. CONCLUSION AND PERSPECTIVES

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28. Conclusions from Zinc Transporter Mutations for Zinc Physiology

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Abstract. The past decade has seen a rapid increase in our understanding of the physiological functions of zinc transporters in the Slc39a (Zip) and Slc30a (ZnT) families in mammals. Remarkably, about half of the 24 putative zinc transporters (6 of the 14 Zip family members and 7 of the 10 ZnT family members) are mutated in a disease and/or have been knocked-out leading to disease phenotypes. Information gleaned from these studies accentuates the surprising diversity of the physiological functions of zinc. Thus far, three mammalian zinc transporters have been reported to be essential for development and viability of the embryo or neonate. Several others become essential first during critical zinc-sensitive periods of development, differentiation and/or function of specific cell-types. Yet other zinc transporters appear to serve ancillary functions in the homeostasis of zinc by participating in its retention or redistribution. Future studies of the effects of mutations in Zip and ZnT family members will be revealing and conditional knockout studies are required to probe other potential cell-specific functions of almost all of the Zip and ZnT family members.

Keywords. disease, gene expression, knockout mouse, mutations, zinc deficiency, Zip, ZnT, zinc transporter

Introduction

The maintenance of zinc homeostasis is critical, and multiple genes modulate the efflux and uptake of this essential metal. In mammals, 24 different genes contribute to these processes. Two superfamilies of mammalian zinc transporters have been identified that belong to the solute carrier (Slc)30a and the Slc39a families [1-3]. Slc30a members, of which 10 have been identified, are named ZnTs and function in zinc efflux and compartmentalization. They are cation diffusion proteins [1]. Members of the Slc39a family, named ZIPs, function in the uptake of zinc and other metals [2-4]. In mammals there are 14 members of the ZIP family most of which can be grouped into one of two subfamilies named subfamily II (3 members) and LIV-1 (9 members). Many of these zinc transporters are expressed in a tissue-specific manner and in specific cellular localizations. In addition, they can display specific changes in cellular localization and stability in response to zinc deficiency or excess [5-8]. Several recent reviews cover this topic in depth [9-13] and a detailed description is given in chapter 8. In the past decade our knowledge of the mammalian zinc transporters has grown dramatically and genetic mutations in ZnT and ZIP family members have begun to provide insight into

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their diverse and often unique physiological functions. Herein, we focus on the members of these families that have been found to be mutated or were purposefully mutated and what that has revealed about their functions.

1. Mutations in Zip (*Slc39a*) family members

1.1 Subfamily II (*Zips1, 2 and 3*)

To date no mutations in members of this subfamily have been linked to a human disease and mouse knockout models suggest that these genes are not essential [11]. In mice, deletion of this entire family of genes does not lead to overt signs of zinc deficiency [14]. However, mice lacking these genes display increased susceptibility to dietary zinc deficiency suggesting that these genes participate in zinc homeostasis, and recent studies of knockout mice provide evidence that their functions can be cell-type specific. Studies of these zinc transporters suggest they function in the retention or redistribution of zinc and not in the acquisition of dietary zinc.

1.1.1 *Zip1* (*Slc39a1*)

The *Zip1* gene is actively expressed in most cultured cell lines and in most organs, however, studies of expression of an eGFP-knockin allele revealed that mouse *Zip1* gene expression is often cell-type specific *in vivo* [15]. For example, *Zip1* expression is high in intestinal stromal cells but not in intestinal enterocytes.

The functions of *Zip1* remain elusive. It is not an essential gene in mice [11, 15]. During the development of prostate cancer in humans, expression of *Zip1* is dramatically down regulated and zinc levels in the cells decrease [16, 17]. This has lead to the speculation that *Zip1* may function as a tumor suppressor and play a primary role in carcinogenesis [18]. However, studies of *Zip1*-knockout mice do not support this hypothesis. The decreased expression of *Zip1* in prostate cancer cells may reflect their dedifferentiation into a cell in which the gene is down-regulated. Studies of *Zip1* and 3-double knockout mice are discussed below.

1.1.2 *Zip2* (*Slc39a2*)

High level expression of *Zip2* is remarkably cell-type specific, occurring in peri-central hepatocytes, keratinocytes and immature dendritic cells [19] as well as in leukocytes of asthmatic infants [20]. The loss of function of this gene renders mice more sensitive to experimental autoimmune encephalomyelitis (Massaki Murakami, unpublished results). Thus, *Zip2* may play a prominent role in the immune system. A polymorphism in the human *Zip2* gene has been associated with carotid artery disease in a cohort of patients, but cause and effect has not been established [21]. Studies of *Zip2*-knockout mice revealed that this zinc transporter not only plays an important role in the adaptation to dietary zinc deficiency during pregnancy but also in the homeostasis of iron in the liver as well as iron and calcium in developing embryo during zinc deficiency [19]. Thus, the physiological functions of zinc transporters can involve interplay between zinc homeostasis and the homeostasis of other essential metals *in vivo*. Similarly the

pharmacological functions of zinc transporters can involve toxic metals as is discussed below.

1.1.3 *Zip3* (*Slc39a3*)

The mouse *Zip1* and *Zip3* genes are co-expressed in many cell-types (e.g. hepatocytes, intestinal stromal cells, kidney ductal epithelium) but *Zip3* expression is also active in Islets of Langerhans, in germ cells in the testes and in mammary secretory epithelial cells [15, 22]. In the mouse brain *Zip1* and *Zip3* are predominantly expressed in the hippocampus and knockout of these zinc transporters protects CA1 hippocampal neurons from glutamate excitotoxicity. This indicates a role for these zinc transporters in the uptake of zinc into the post synaptic neuron [23]. Studies of *Zip3*-knockout mice revealed an interesting function in zinc reuptake from the alveolar lumen in the lactating mammary gland. Expression of *Zip3* is active in the mammary secretory cells and *Zip3* localizes to the plasma membrane of these cells where it functions to maintain the zinc levels in the milk by reuptake [22]. Knockout mice transiently accumulate more zinc in the milk. Taken together current data support the conclusion that Zip subfamily II zinc transporters (Zips 1-3) function in the redistribution and/or retention of zinc rather than its acquisition from the diet [15, 24].

Future studies of tissue-specific knockout models of *Zips 1, 2 and 3* are required to understand the diverse functions of this family of zinc transporters.

1.2 *LIV-1* subfamily (9 members)

1.2.1 *Zip4* (*Slc39a4*)

Interesting functions of several members of the LIV-1 subfamily have been revealed by gene mutations and transgenic approaches. In humans, mutations in the *Zip4* gene cause the pseudo-recessive genetic disorder acrodermatitis enteropathica [25-27]. Impaired absorption of zinc by the intestine causes symptoms of this disease to appear after weaning in humans [28] and multiple mutations have been mapped to the human *Zip4* locus. This disease is lethal unless treated by supplementation with excess dietary zinc. The expression of this gene is dynamically regulated by zinc in the intestine and visceral endoderm in mice [29] and the mouse *Zip4* gene is essential during early embryonic development. Homozygous knockout embryos die during morphogenesis [30] and *Zip4* expression in the preimplantation mouse embryo is active in the visceral endoderm at the egg cylinder stage which demonstrates an essential function of *Zip4* in the uptake of zinc into the mouse conceptus soon after implantation. *Zip4* heterozygosity is also teratogenic and embryotoxic in mice and maternal *Zip4* heterozygosity renders mice hypersensitive to zinc deficiency during pregnancy. Similarly, it has recently been found that *Zip4* heterozygosity in humans can cause disease [27]. Thus, acrodermatitis enteropathica should be considered a pseudo-recessive disorder. Whether *Zip4* serves functions in addition to the absorption of dietary zinc remains to be determined. In that regard, aberrant high level expression of *Zip4* has been found in pancreatic and hepatocellular carcinomas in humans and mice and may also occur in many other types of cancers, where it can enhance cell cycle and cell migration and repress apoptosis [31-34].

1.2.2 *Zip8* (*Slc39a8*)

Recent studies of Zip8 reveal a function in cadmium toxicity as well as zinc transport [35-37]. This gene was mapped to the *Cdm* locus in mice, which is associated with differences in cadmium-induced testicular necrosis between inbred strains. Zip8 functions as a zinc/bicarbonate symporter and cadmium-sensitive mice actively express *Zip8* in the testes [35, 38]. Transgenic expression of the *Zip8* locus from cadmium-sensitive mice rendered cadmium-resistant mice sensitive to the testicular toxicity of cadmium as well as to nephrotoxicity [39]. Differences in the promoter region of this gene from sensitive versus resistant mice appear to account for the cell-specific expression leading to cadmium sensitivity. These studies reemphasize that function of the ZnT and Zip family members in mammals can involve metals in addition to zinc and may be of pharmacological significance.

1.2.3 *Zip13* (*Slc39a13*)

Mutations in the *Zip13* gene have recently been found to cause a unique form of Ehlers-Danlos syndrome [40]. Ehlers-Danlos syndrome is a group of heritable disorders of connective tissue development [41] and patients with a newly recognized subtype named spondylocheiro dysplastic Ehlers-Danlos syndrome display, among other problems, hyperelastic and easily bruised skin, joint hypermobility, skeletal dysplasia, and growth retardation. Affected siblings show progressive short stature and retarded growth beginning in the first year. Amino acid deletions within transmembrane domain III [42] and a non-conservative amino acid substitution within transmembrane domain II [40] have been found in these patients. Studies of *Zip13*-knockout mice suggest that these are loss-of-function mutations and homozygous *Zip13*-knockout mice recapitulate the phenotypes of spondylocheiro dysplastic Ehlers-Danlos syndrome [40]. These mice display defects in the maturation of osteoblasts, chondrocytes, odontoblasts and dermal fibroblasts apparently due to impaired bone morphogenic signaling. Thus, *Zip13* serves a highly cell-specific function during development of bone, teeth and connective tissue.

1.2.4 Genetic studies of other LIV-1 family members

The physiological functions of many mammalian ZIP family members remain to be determined. However, the *fear-of-intimacy* gene in *Drosophila* encodes a LIV-1-like protein that can function as a zinc transporter and that is essential during early development of the fly [43, 44]. This gene controls germ cell migration and gonad formation which suggests a key role for zinc in these processes. Similarly, mutations in the *catsup* gene, a member of the *Slc39a* family, leads to over-expression of dopamine [45]. Catsup is a negative regulator of tyrosine hydroxylase activity and mutations in it cause a loss-of-migration phenotype leading to abnormalities throughout development [46]. Morpholino knockdown of zebrafish *zLiv-1* revealed a function in epithelial-mesenchymal transition in the gastrula [47]. *zLiv-1* is a downstream target of STAT3, and is essential for the nuclear localization of the zinc-finger protein Snail, which regulates epithelial-mesenchymal transition. This suggests that other mammalian zinc transporters in the LIV-1 subfamily will likely be found to have important cell-type specific physiological functions.

2. Mutations in ZnT (Slc30a) family members

The functions of several members of the ZnT family have been deduced from studies of knockout mice and *Drosophila*, but thus far only ZnT2 and ZnT8 has been shown to be mutated or polymorphic in human disease, respectively. ZnT4 loss-of-function in mice causes the lethal milk mutant.

2.1 ZnT1 (*Slc30a1*)

The mouse *ZnT1* gene is essential during early development and may be involved in the transfer of zinc into the conceptus [1, 48]. Targeted deletion of *ZnT1* results in early embryonic lethality in homozygous knockout mice [48]. High levels of *ZnT1* mRNA are found in the maternal deciduum and in the visceral yolk sac surrounding the mouse embryo, which suggests that this protein is involved in the transfer of maternal zinc into the uterine and embryonic environments [48]. This lethality has precluded further studies of the functions of ZnT1 in the adult mouse and no conditional knockout studies have been reported.

Recent studies of the *Drosophila* ZnT1 orthologue, dZnT1, provided further clues as to its function [49]. RNA interference knockdown of dZnT1 also causes developmental arrest when dietary zinc is restricted. Thus, ZnT1 serves an important function during development in mice and *Drosophila*. dZnT1 is localized to the basolateral membrane of the midgut enterocytes and gut-specific silencing causes lethality when zinc is deficient in adult flies. Human ZnT1, but not ZnT7 or ZnT4, could complement the loss-of-function of dZnT1 in the gut [49]. These studies support the notion that ZnT1 functions in dietary zinc absorption by pumping zinc out of the enterocytes across the basolateral membrane. During development it is likely that ZnT1 also functions by transferring zinc into the embryo from the mother in mammals or from the yolk in flies. Conditional knockout approaches will be required to discern other functions of ZnT1 as can also be said for most of the other ZnT family members.

An unexpected and very interesting finding regarding ZnT1 function was recently reported [50]. Susceptibility to certain human papillomaviruses is dependent on mutations in either of two genes (EVER1 and EVER2). EVER1 and EVER2 proteins were shown to form a complex and interact with ZnT-1. EVER and ZnT-1 proteins both affected intracellular zinc distribution and EVER2 inhibited free zinc influx into nucleoli. Oncoprotein of genital (HPV16) genotypes was shown to bind to the EVER:ZnT-1 complex and block their negative regulation of zinc uptake. These studies reveal EVER proteins as modifiers of ZnT1 function and suggest that zinc balance mediated by these proteins may protect against papilloma virus infections.

2.2 ZnT2 (*Slc30a2*) and ZnT4 (*Slc39a4*)

ZnT2 expression is most robust in tissues with unique zinc requirements, such as the mammary and prostate glands. Recently, mutations in *ZnT2* were discovered in a women whose nursing baby displayed symptoms of transient neonatal zinc deficiency. Transient neonatal zinc deficiency is a very rare disorder that affects newborn children. At least in some cases, this syndrome is caused by the inability of the nursing mother to provide sufficient zinc in breast milk during the first few months post-partum [51]. *ZnT2* was identified as the gene responsible for transient neonatal zinc deficiency in one family [51]. Mothers of afflicted children were heterozygous for a missense

mutation in exon 2 therefore this is an autosomal dominant disease. This mutation resulted in a histidine-54 to arginine non-conservative substitution in the cytoplasmic amino-terminus. This residue (histidine-54) is well conserved among ZnT family members but is located in a non-structured region of the protein that is apparently not involved in zinc binding or transport. This mutation may influence protein localization, folding or dimerization with partner proteins. ZnT2 is localized to the vesicular compartment in the mammary gland and its abundance is regulated by prolactin signaling via the Jak2/Stat5 pathway in cell culture [52, 53]. These data support the conclusion that ZnT2 functions in the secretory pathway of human mammary epithelial cells to add zinc to milk during the first months after parturition.

Mutations in the mouse *Znt4* gene and human *Znt2* gene have both been associated with a loss or reduction, respectively, of zinc in milk [51, 54]. The lethal milk (*lm*) mutation in mice leads to insufficient zinc in milk to support the suckling pups. The *lm* mouse has a nonsense mutation at arginine-297 in the *ZnT4* gene and is an autosomal recessive disease [54]. One reason the phenotypes of several zinc transporter mutants are readily recognized is that zinc requirements for rapidly growing embryonic, fetal and newborn mammals is particularly high. These stages of development are most susceptible to the effects of zinc deficiency and the phenotypes are often visually accessible. The studies of ZnT2, ZnT4 and Zip3 demonstrate the complexity of how zinc levels in milk are controlled. This is perhaps not unexpected given the critical importance of providing sufficient zinc to newborn babies.

2.3 *ZnT3* (*Slc30a3*)

The *ZnT3* gene is expressed in neurons and is important for the acquisition of zinc in synaptic vesicles. *ZnT3*-knockout mice lack zinc in these vesicles [55, 56]. Zinc-enriched neurons in mammals are primarily glutamatergic and located in hippocampus, amygdala and neocortex. No major physiological and behavioral changes were found in studies comparing young *ZnT3*-knockout mice with wild-type littermates [56], but a recent study revealed age-dependent deficits in learning and memory at 6 months of age in the *ZnT3*-knockout mice [57]. These deficits were associated with changes in hippocampal proteins involved in learning and memory and appear to mimic Alzheimers disease. Thus, ZnT3 may function, in part, to support development/maintenance of neurons and/or formation/maintenance of proper neuro-circuits by sequestering zinc within specific neurons.

It has been suggested that zinc released into the synaptic cleft acts as a neuro-modulating agent on postsynaptic receptors and, in addition, it has been hypothesized that neuronal damage following traumatic brain injury, ischemia and seizures is exacerbated by presynaptic zinc that is released and subsequently taken up by postsynaptic neurons leading to cell death. However, *ZnT3*-knockout mice are more prone to kainic acid induced seizures and a recent study revealed that traumatic brain injury in *ZnT3*-knockout mice initially causes more neuronal damage compared with wild-type mice [56]. This suggests that vesicular zinc may not cause neurological damage in this model and that the vesicular zinc has other functions than neuro-degenerative ones.

Expression of the *ZnT3* gene is not restricted to neurons and has been documented in pigmented retinal epithelium [58] and in β -cells of the pancreas [59]. *ZnT3*-knockout mice have recently been shown to display altered glucose metabolism during

β -cell stress [59]. Thus, the physiological functions of ZnT3 appear to be quite diverse and centered on the uptake of vesicular zinc.

2.4 ZnT5 (*Slc30a5*) and ZnT7 (*Slc30a7*)

Complexes containing ZnT5 and 6, as well as complexes containing homo-oligomers of ZnT7 function to transport zinc into the secretory pathway facilitating the activation zinc-requiring enzymes such as alkaline phosphatase [60, 61]. Homozygous deletion of *Znt5* results in poor growth, osteopenia, low body fat, muscle weakness, and male-specific cardiac death [62]. Thus, ZnT5 functions to transport zinc into the secretory compartment, a process critically important for many organs, as is expected [60]. *Znt7*-knockout mice also fail to thrive, have low body fat and show diminished acquisition and distribution of dietary zinc [63] perhaps also reflecting impaired zinc transport into the Golgi apparatus [64]. Thus, these knockout models reveal that transfer of zinc into the secretory pathway by ZnT5 and/or ZnT7 is particularly critical for proper post-natal growth and the formation and/or maintenance of body fat within the physiological context of the animal. Mutations in these genes appear to dramatically affect metabolism. ZnT5 may also play an important role in the male heart. As mentioned above for most of the ZnT and Zip family, conditional knockout studies are required to further investigate tissue-specific functions of these proteins. It will also be important to identify modifier genes which may change the phenotypic expression of zinc transporter mutations.

2.5 ZnT8 (*Slc30a8*)

The *ZnT8* gene functions in insulin processing, crystallization and secretion by its ability to put zinc into insulin secretory granules in the pancreatic islets [65, 66]. Insulin co-crystallizes with zinc. ZnT8 is localized on the membranes of insulin secretory vesicles, but is also found on secretory vesicles in other endocrine organs [67]. ZnT8 has been identified as a major autoantigen in patients with diabetes and autoimmunity against ZnT8 can be prognostic of Type II and Type I diabetes [68-70]. Genome wide association studies have identified a common polymorphic form of ZnT8 (R325W) (rs13266634) which strongly correlates with the development of Type I diabetes and with reduced first-phase secretion of insulin in Type II diabetics [71]. It is interesting to note that the R325W variant is associated with resistant to posttransplantation diabetes in renal allograft patients [72]. ZnT8 appears to function as a homodimer and amino acid 325 is predicted to be at the dimer interface [73]. The W325 polymorphic variant has lower zinc transport activity.

Studies of *ZnT8*-knockout mice and β -cell specific *ZnT8*-knockout mice have recently been reported [73-76]. In the absence of ZnT8, insulin crystallization is disrupted and insulin release is diminished *in vivo* [74-76]. *ZnT8*-knockout mice appear to have normal glucose homeostasis when young unless fed a high-fat diet, in which case they become glucose intolerant and islets from these mice become less responsive to glucose [74]. *ZnT8*-knockout mice show age, diet and sex dependent abnormalities in glucose metabolism, insulin secretion and body weight [73]. *ZnT8*- β -cell-specific knockout mice are glucose-intolerant, have reduced β -cell zinc accumulation and atypical insulin granules [76]. They also display reduced first-phase glucose-stimulated insulin secretion, reduced insulin processing enzyme transcripts and increased proinsulin levels. Thus, ZnT8 is expressed in a subset of cell-types where it

functions to sequester zinc into secretory vesicles. In the β -cell, ZnT8 functions to put zinc into the insulin secretory granules. The functions of ZnT8 in other endocrine cell-types remain to be determined but the availability of mice with floxed ZnT8 alleles will facilitate such studies [76].

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29. Where We Are and Where to Go in Zinc Research?

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Abstract. The knowledge about zinc has increased dramatically during the last two decades. Especially the discovery of specific zinc transporters has pushed the field. However, we are just at the beginning of a new century for this trace element, since it becomes more and more obvious that zinc is regulating a multitude of cellular processes.

Keywords. RDA; Zinc homeostasis, Zinc deficiency; Zinc Supplementation

Introduction

The 27 chapters have shown that our knowledge about zinc has increased dramatically, but is still limited. In chapter 28 Glen Andrews summarized that our knowledge about the zinc transporters is also just at the beginning, since knockout models often differed from the estimated effects. Therefore we must evolve a master plan of things to be investigated to complete our knowledge about zinc.

First we have to answer the very simple question about the recommended daily intake of zinc, in order to clear the zinc status by the daily food supply. Today the data would even change if one uses the recommendations of another country. This recommendation must be on the basis of available zinc in the food supply, since this varies dramatically (see chapter 3). The second important point is to analyze the available zinc supplements and compare their , since so far no comprehensive study exists answering this simple question. With this knowledge the comparability of zinc supplementation studies will increase and we will learn much more about the right concentration for the treatment of different diseases. This will also improve the use of zinc in developing countries and may help us to prevent more childhood diseases.

For the molecular research it will be necessary to synthesize specific zinc probes which will allow to exactly determine the concentration of free zinc in different cellular compartments. Maybe these probes will also help us to define the real zinc status in man, since our clinically used measurement of serum zinc by atomic absorption is a bad parameter to analyze the zinc status. Serum zinc is just 0.1% of the body zinc content and a very flexible pool. Since even the zinc concentration in leukocytes is considerably higher, this pool may not reflect the real zinc status of a person. Actually

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we have problems to analyze the zinc homeostasis in patients and to rebalance the zinc homeostasis due to the lack of an accurate measurement of the zinc status.

The understanding of zinc homeostasis will be the next big goal, since as we have seen in the first chapters, the zinc homeostasis regulates a number of cellular processes. This will directly lead to a better understanding of the role of zinc in different organ system. To measure and to rebalance zinc homeostasis may become a favorite treatment for family doctors as one could estimate from the large number of people prone to marginal zinc deficiency worldwide.

The effects may be the reduction of infectious diseases in children, especially in the developing countries, and elderly, especially in the industrial countries. Furthermore, the maturation of children as well as their cognitive development may improve. In the elderly the chronic inflammatory processes may be decelerated as well as the cognitive decline if we are able to understand zinc homeostasis in these processes right.

Lastly zinc homeostasis may help us to understand the complex alterations in the cells during cancer. We have seen that zinc increases in mamma carcinoma, whereas it decreases in prostate carcinoma. However, in both cases the zinc homeostasis is dramatically disturbed and a rebalance may be an advantage for the patients. The general problem that zinc deficiency and zinc overload often show similar effects favors the theory that zinc homeostasis is a flexible and regulatory system in the body. Multiple of cellular processes can be altered just by altering the cellular concentration of zinc. Therefore the alteration of zinc is an acute phase response, which could be already observed in infectious diseases. In the future we may understand the regulatory function of different zinc concentrations as a simple mechanism of the organism to alter the reactivity of different organ system by one factor.

Hopefully this book will give an impression of the current knowledge of zinc and the requirement of more research in this field to establish zinc as a useful and cheap drug in medicine.

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